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Enzyme activity assay for horseradish peroxidase encapsulated in peptide nanotubes

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ABSTRACT

Encapsulation of horseradish peroxidase (HRP) inside a peptide nanotube (PNT) was demonstrated and its activity was measured. Enzyme assay verified that 0.16 μg of the enzymes were encapsulated in 1 mg of PNTs. The encapsulation was also verified with TEM, UV–vis spectroscopy, and FTIR. The activity of the encapsulated HRP was examined for thermal stability, long-term storage stability, and resistance to a denaturant. They showed good storage stability, retaining its activity up to 90%, while the free HRP lost 50% of its activity over the course of 18 days. At 55 °C, the encapsulated HRP activity remained 20% higher than that of the free HRP. With the denaturant, guanidinium hydrochloride (GdmHCl), the encapsulated HRP activity was maintained around 10% higher than the free HRP. This result proves that the encapsulation of HRP inside the PNT may be an effective way to keep the enzyme activity stable in various environments.

1. Introduction

When using enzyme reactions in various industrial applications, retention of enzyme activity is often an important issue that should be addressed for successful operation [1,2]. Enzymes lose their activities, a phenomenon known as deactivation, due to a result of several factors or combinations of the following: heat, pH, solvent, surfactant, and heavy metals [3]. Due to deactivation, stabilization of enzymes is a crucial requirement for successful performance in applications such as wastewater treatment, drug delivery, tissue engineering, bio-fuel cells and biosensors [4,5].

Enzyme stabilization is frequently achieved by immobilization, entrapment, or encapsulation in inorganic or organic structures [6]. Enzyme modification with dendron macromolecules and amino acids was used to improve HRP stability [7,8]. A number of bio-inspired materials for immobilization of enzymes have been investigated to improve the enzyme stability and regenerability on a wide range of applications. Recently the nano-structure of peptides in the form of a fibril, sphere, or tube has been recognized as a potential encapsulation approach. For example, diphenylalanine peptide (L-Phe-L-Phe; FF) and its derivatives were effectively used to form three-dimensional (3-D) nanostructures [5]. PNT consists of the diphenylalanine, a short peptide that would be easier to design and synthesize. It was known that electron transfer between spatially aligned aromatic (phenyl) components could contribute to the electrochemical conductivity [9]. So, this property would be useful in the development of bioelectronics and biosensors.

Compared with other encapsulation materials, they offer a highly biocompatible environment and do not require any harsh conditions to synthesize them [10–12]. A peptide nanotube (PNT) has great potential as a biocompatible encapsulating agent that can provide the advantage of good retention of enzymes and enhanced enzyme activity [4]. Peptide-based nanostructures become biocompatible since they are composed of peptides, which are a group of amino acids. The amino acids as the natural and biocompatible compounds could be nontoxic with HRP [8]. Therefore, the use of PNT is attractive for applications such as biosensors, drug delivery and tissue engineering [13]. It turned out that use of the biocompatible dipeptide can reduce the cytotoxic effects [14]. The successful encapsulation of enzymes with a PNT requires the adsorption of enzymes on the inner surface of the tube wall with its active sites facing away from the wall surface of the PNT, and little or no leaching of the enzymes [15]. The PNT could provide the large inner surface that makes the enzymes to have an easy access since the pore diameter of PNT is relatively large compared to other nanostructure such as silica nanostructure (MCM-41, SBA-15, and MCF) [16]. Although the walls of PNTs are not impermeable, most diffusion of enzymes and other molecules occurs though the two holes in the end of a tube.

In the present study, diphenylalanine PNTs were synthesized as an encapsulation support for the enzyme, horseradish peroxidase (HRP), to enhance thermal and storage stabilities of the enzyme. The

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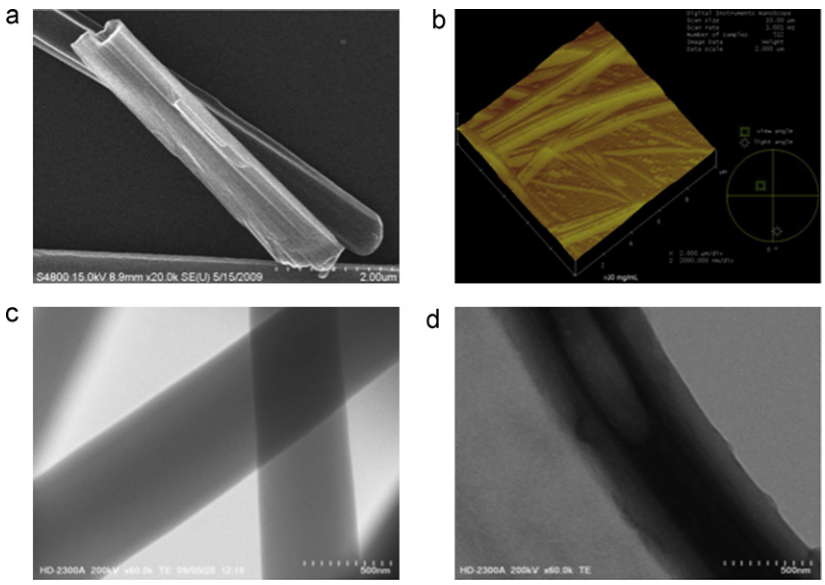

Fig. 1. (a) SEM image of the PNT, (b) AFM image of the PNT, (c) STEM image before the encapsulation of the HRP inside the PNT, and (d) after the encapsulation.

activity of the encapsulated enzyme was measured and compared with that of the free enzymes.

2. Materials and methods

2.1. Materials

Diphenylalanine (Phe-Phe) was purchased from Bachem Bioscience. 1,1,1,3,3,3-hexafluoro-2-propanol, horseradish peroxidase, bovine serum albumin, ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)), H_2O_2, K_2HPO_4, KH_2PO_4 and guanidinium hydrochloride (GdmHCl) were obtained from Sigma. All the solutions were prepared with double-distilled water.

2.2. Formation of PNT

The diphenylalanine (Phe-Phe) peptide monomer was dissolved in a highly volatile solvent of 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) at a concentration of 100 mg/ml. The self-assembly of the peptide nanotubes was carried out at the optimal concentration of 2 mg/ml of the monomer. It was known that the concentration is a crucial factor for forming self-assembled PNTs [17].

2.3. Encapsulation procedure

Encapsulation of HRP inside the PNT was performed in the manner described as follows: the PNT solution of 1 ml in a glass vial was placed in an oven at 35 °C to dry the PNT completely. One milliliter of 50 mM phosphate buffer solution (PB, pH 5.8) containing HRP at a concentration of 0.32 μg/ml was dropped into the vial that contained the dried PNT. It was mixed using a vortex mixer subsequently. For the encapsulation procedure, the enzyme was incubated in a shaker at 5 °C, 120 rpm for a week. To separate the PNT from the solution, the micro centrifugation was carried out at 16,000 × *g* for 10 min. After separating the PNT from the solution, the PNT was carefully and thoroughly washed three times with the PB solution to remove the excess HRP.

2.4. Electron microscopies

Samples for the STEM (scanning transmission electron microscopy) were prepared by dropping 5 μl of the PNT solution on 200 mesh copper grids and then the samples were dried completely at room temperature. The STEM (Hitachi HD-2300, Japan), used for the morphology characterization of the PNT and the encapsulated HRP with the PNT, was operated at 200 kV acceleration voltages. Scanning electron microscopy (SEM) (Hitachi S-4800, Japan) was operated at 15 kV acceleration voltages. Atomic force microscopy (AFM) (Veeco Multimode Nanoscope IIIa, USA) in the tapping mode was also used for characterization of PNTs. All samples were air-dried and then analyzed by these instruments.

2.5. Spectroscopies

UV–vis spectroscopy (Shimadzu UV-2450) and FTIR spectroscopy (Varian FTS4000) were used to verify the encapsulation of the enzymes inside the PNT. For FTIR spectroscopy attenuated total reflectance Fourier transform IR (ATR-FTIR) spectroscopy was used to identify the conformational structure change of HRP encapsulated inside the PNT. The Varian FTS4000 system consists of UMA 600 FTIR microscope with a focal plane array (FPA) image detector, and a semispherical germanium crystal. To obtain a sufficient signal to noise ratio and resolution of spectra, data were collected in the range of 600–4000 cm^{-1} with 256 scans per sample and a resolution of 2 cm^{-1}. All spectra were processed using built-in software.

2.6. Enzyme activity assay

The enzyme activity of the HRP encapsulated inside the PNT was determined using the ABTS assay for peroxidase. The HRP catalyzes 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), ABTS, in the presence of hydrogen peroxide to form an oxidized ABTS. For enzyme assay of the samples, 2.9 ml of a 9.1 mM ABTS solution and 0.1 ml of 0.3% (w/w) of H_2O were mixed with a 2 mg sample of the PNTs containing HRP and also with 0.05 ml of the free HRP solution. For a blank, 0.05 ml of PB solution with 0.25 (w/v) of bovine serum albumin (BSA) was used. Absorbance at 405 nm was measured for 20 min.

2.7. Leakage test

The encapsulated HRP into the PNT was mixed with a PB solution and incubated at 4 °C for 30 min. Then the encapsulated HRP into the PNT was removed and the remainder solution was tested the enzyme activity using the ABTS assay. The leakage test was successively conducted five times.

3. Results and discussion

The morphology of the PNT was investigated using SEM and AFM. A SEM image is shown in Fig. 1(a). The PNT is a hollow nanostructure, in which a space available for the encapsulation of enzymes. The AFM image in Fig. 1(b) shows the PNTs with different diameters. Analyses of both images of the obtained PNT indicate a broad range of diameter, ranging from 10 nm to few micrometers.

The encapsulation of HRP inside the PNT was confirmed by STEM as shown in Fig. 1(c) and (d). STEM images of the PNT exhibit its hollow space in the center of the tube. Compared to the hollow PNT before encapsulation shown in Fig. 1(c), the layer of HRP appears in darker areas on the inner surface of the PNT as in Fig. 1(d) [15,18]. It is observed that the thickness of the PNT wall was 20–90 nm and the diameter was several nanometers to micrometers. These results represent that the inner wall of the PNT was coated with the HRP.

Even though we do not have clear evidence as to how the HRP was adsorbed on the inner surface of the PNT, it is thought that it happened through a hydrophobic interaction between HRP and

the PNT. It was reported that the hydrophobic residues of lipase adsorbed on the inner surface of a PNT [15]. When *cytochrome c*, one of the peroxidative heme proteins, was encapsulated inside pores of mesoporous silicates, the higher enzyme activity of peroxidase was reported to be achieved through adsorption of the enzymes. Yang et al. reported that at a low concentration, nanoparticles could be encapsulated inside supports through capillary effect [19].

The activity of the free enzymes in the supernatant was measured by the ABTS enzyme assay to estimate the amount of encapsulated enzymes. The amount of the encapsulated HRP was estimated at room temperature by subtracting the HRP concentration in the supernatant from the initial concentration of the HRP. In the leakage test, it was shown that the encapsulated HRP has strong affinity for the inner surface of the PNT as the retention of the enzyme was observed to remain at 0.16 μg HRP/mg PNT after up to 5-time washing. The leakage experiment showed that no protein was detected in a PB solution using the ABTS assay. This result proves the enzyme adsorption was irreversible at a low concentration of the HRP. There are a number of possible interactions for the HRP adsorption onto the PNT surfaces, including hydrogen bonding, hydrophobic, and electrostatic interactions. In most cases, high enzyme adsorption is observed on hydrophobic surfaces [20]. Moreover, it was considered that the immobilization of the enzyme could be achieved by amino acid groups, which are of the Lys amino residues [1]. In the present case, amino acid residues (Lys) near the HRP surface would possibly facilitate the adsorption of the HRP on the surface of the PNT containing carboxyl groups. First, amino groups (NH_2) of lysine of the HRP could interact with carboxyl groups of the PNT by hydrogen bonding. Second, the hydrophobic interaction between the hydrophobic domain of HRP and the hydrophobic phenyl groups of PNT would result in the HRP adsorption. Thus, the HRP would interact with the PNT by hydrogen bonding and/or hydrophobic interaction.

UV–vis spectroscopy is a versatile tool for analyzing the characteristic structure of enzymes. The peak position at 402 nm of the Soret absorption of the heme iron in a HRP molecule provides the information about HRP conformation when it is adsorbed on a support. When HRP is denatured, the Soret band shifts or disappears [21,22]. Fig. 2 verifies that no peak shift occurred when the enzymes were adsorbed in the PNT. This result demonstrates that the HRP encapsulated inside the PNT has a secondary structure identical to the native state of HRP in the solution. In other words, the proper orientation of the active site of the enzyme was kept during the adsorption. If the active site of the enzyme, the heme group, was denatured during the adsorption, the absorption band would have shifted.

The ATR-FTIR result in Fig. 3 also verified successful immobilization of HRP inside the PNT. The amide I band (1700–1600 cm^{-1}) of HRP, which is mainly caused by C=O stretching vibrations of peptide linkages, appears at 1643.12 cm^{-1}. The peak at 1538.20 cm^{-1} shows the characteristic of amide II (1600–1500 cm^{-1}), which is caused by a combination of N—H in plane bending and C—N stretching vibration of the peptide groups [23,24]. For the spectrum of the PNT, the PNT exhibited a strong peak at 1687.64 cm^{-1} in the amide

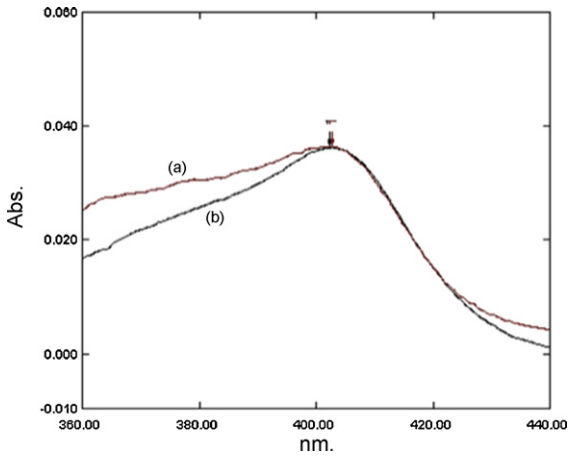

Fig. 2. (a) UV–vis spectra of the HRP in the bulk phase. (b) UV–vis spectra of the mixture of the HRP in the PNT.

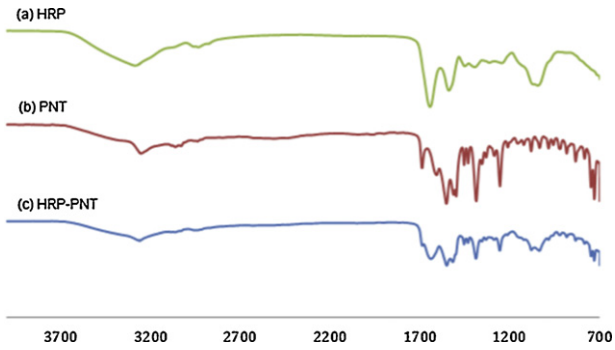

Fig. 3. ATR-FTIR spectra of (a) free HRP, (b) PNT and (c) the encapsulated HRP into the PNT.

I band region. The peak can be attributed to a predominant β-sheet conformation [25]. Multiple bands between 1400 and 1600 cm^{-1} appearing in all spectra indicate the presence of aromatic rings of diphenylalanine [26]. The spectrum of the HRP/PNT combination having amides I and II bands of 1637.71 cm^{-1} and 1550.32 cm^{-1} is almost the same as that of the native HRP. The similarities of those spectra indicate that the native secondary structure of HRP was retained in the HRP-PNT. The biocompatibility of PNT seems to support conformational stability of HRP during and after the enzyme immobilization. Therefore PNTs may provide a novel template for effective enzyme encapsulation or immobilization.

Thermal stabilities of the encapsulated HRP inside the PNT and the free HRP in the bulk phase are compared in Fig. 4(a). The activity variation of the HRP was observed at a temperature range from 25 to 55 °C. The samples at each temperature were held at the specific temperature for 10 min before the measurement was taken. Fig. 4(a) shows the percentage of activity of the HRP as a function of temperature. The activity of the free HRP at 55 °C was 60% of

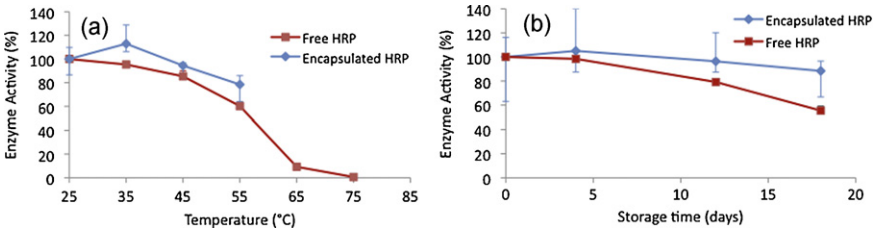

Fig. 4. (a) Thermal stability of the encapsulated HRP. (b) Storage stability of the encapsulated HRP.

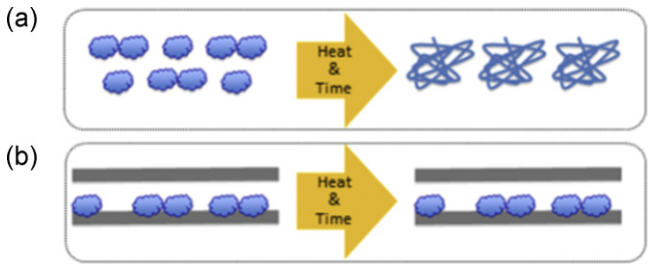

Fig. 5. Schematics of possible explanation how the enzyme can be protected by the PNT encapsulation at high temperature and for an extended storage time. (a) Free HRP. (b) Encapsulated HRP.

the initial activity at 25 °C, whereas the encapsulated HRP retained an activity of about 80% at 55 °C. The experimental results demonstrate that the effect of higher temperatures becomes less serious for the encapsulated HRP inside the PNT.

In Fig. 4(b), the enzyme activities of the free HRP and encapsulated HRP were compared at different time intervals. The samples were incubated in a refrigerator at 4 °C until they were used at room temperature for the each time interval, 4, 12, and 18 days. The free HRP lost about 50% activity, while retaining 54% (±4) activity after 18 days. The encapsulated HRP retained its stability of 88% (±10) for 18 days.

It is thought that the PNT encapsulation provides a rigid external backbone that protects the enzyme molecules from the temperature damage. For the delaying of deactivation with time, the encapsulation seems to prevent the HRP from denaturing because a conformational change of the enzyme is hard to take place inside the cylindrical space of PNT. Fig. 5 illustrates the protective character of the PNT against heat and time. However, above 60 °C, the self-assembled PNT started to disassemble in the PB solution, even though it was reported that the PNT could be stable and maintain the structure up to 150 °C in a dry condition [27]. It is thought that pH of the PB solution affected the hydrogen-bonding of the PNT to facilitate the dissemblance at high temperatures in a liquid phase.

It was reported that guanidinium hydrochloride (GdmHCl) may be used as a denaturant of enzymes, causing conformational changes in enzymes and a loss of enzyme activity [28]. Use of a denaturing agent is a useful method to prove the stability of enzymes confined in the PNT nanostructure. Ikemoto et al. reported that HRP encapsulated inside the cavity of nanoporous silica showed a slightly higher activity than free HRP when denaturing agents such as GdmHCl and urea were applied [29]. In a bulk phase, even the presence of low concentrations of GdmHCl altered the tertiary structure of HRP [28].

In this study, the stability of free and encapsulated HRP against 100 mM GdmHCl was compared. Then each sample was stored for one week at 20 °C and then its enzyme activity was measured again. The activity of the free HRP was 80% of the initial activity, whereas the encapsulated HRP retained an activity of about 90% after one week of incubation with GdmHCl. It turned out that the enzyme activity of the encapsulated HRP was observed to be an average of 10% higher than that of the free HRP. Also, no destruction of the PNT was observed with 100 mM of the GdmHCl at pH 5.8. This result agrees with other reports that the self-assembled nanostructure of dipeptides was stable in the presence of GdmHCl and acidic conditions [30].

It could be possible that enzyme aggregation takes place so that the stability of the enzyme can be enhanced [31]. In the present case, however, it is mostly thought that a rigid backbone of the PNT can protect HRP from denaturation and enzyme aggregation because aggregation of enzymes is generally known to reduce the enzyme activity [32].

4. Conclusions

To the best of our knowledge, it was the first time that the PNT consisted of dipeptides was utilized as an encapsulation agent for enzymes. The enhanced activity of HRP inside the PNT was higher than that of the free HRP in the solution in terms of the thermal effect, storage time and a denaturing agent. A relatively simple synthesis method is another advantage of using the PNT. Overall, the enhanced stability of the HRP conjugated with the PNT offers great potential to developing novel biocatalyst and biosensor systems.

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