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Fabrication and Application of Enzyme-Incorporated Peptide Nanotubes

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Abstract

Enzyme engineering is a fast-growing field in the pharmaceutical and food markets. For those applications, various substrates have been examined to immobilize and stabilize enzymes. In this report, we examined peptide nanotubes as supports for enzymes. When a model enzyme, *Candida rugosa* lipase, was encapsulated in peptide nanotubes, the catalytic activity of nanotube-bound lipases was increased 33% as compared to free-standing lipases at room temperature. At an elevated temperature, 65 °C, the activity of lipases inside the nanotubes was 70% higher than free-standing lipases. The activity enhancement of lipases in the peptide nanotubes is likely induced by the conformation change of lipases to the open form (the enzymatically active structure) as lipases are adsorbed on the inner surfaces of peptide nanotubes.

INTRODUCTION

Enzymes are versatile biocatalysts that control specific chemical reactions effectively in vivo and in vitro (1–3). To make enzymes cost-effective, long-lived, and highly active, supports, such as sol–gels (4, 5), polymer membranes (6), silica (7, 8), and zeolites (9, 10), have been utilized to immobilize enzymes. While there are supports that enhance enzymatic activity, lifetime, and thermal stability (4, 6, 10, 11), some supports also decrease the lifetime and the activity of enzymes (8, 12). Nanometer-scale materials have potential to serve as superior enzyme supports due to their large surface-to-volume ratios in comparison with traditional macroscale materials while the reduction of substrate size may induce the deactivation and the desorption of enzymes in the processes of enzymatic reactions (12). Recently, the catalytic activity and the thermal stability of enzymes were reported to be enhanced when they were immobilized on magnetic nanoparticle supports (11, 13). To evaluate the effectiveness of nanoscale enzymatic supports, more studies in various types of nanosupports are desirable.

In this report, we explored a new nanoscale support, peptide nanotube, to immobilize enzymes inside the nanotubes. Peptide nanotubes have an advantage to immobilize enzymes on the nanotube surfaces with a simple incubation process via hydrogen bonding between amide groups of the nanotube and the complementary functional groups of proteins (14, 15). It is also advantageous that the adsorption of enzymes can be limited inside the peptide

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nanotubes via capillary effect (16). Previously, the coating location of peptides and nanocrystals, inside or outside the nanotubes, was controlled by optimizing concentrations of adsorbents and functionalities of nanotube surfaces (16, 17). In this report, a *Candida rugosa* lipase was chosen as a model enzyme to test the peptide nanotube support because the *Candida rugosa* lipase has previously shown enhancement of the enzymatic activity on other supports (11, 13). The enzymatic activity of *Candida rugosa* lipases inside the peptide nanotube was enhanced not only at room temperature but also at elevated temperatures as compared to free-standing lipases.

EXPERIMENTAL PROCEDURES

First, 10 mM of bis(*N*- α -amido-glycylglycine)-1,7-heptane dicarboxylate, bolaamphiphile peptide, was self-assembled into peptide nanotubes in a pH 4.5 citric acid/NaOH solution. Methods for the bolaamphiphile peptide synthesis and the nanotube self-assembly are described elsewhere (18, 19). After the peptide nanotubes were washed with deionized water several times, 200 μ L of nanotubes in a phosphate buffer solution (pH = 7.4) was mixed with 1–20 μ L of *Candida rugosa* lipase solutions, respectively. The enzyme in the peptide nanotube was protected during the incubation time of a week by keeping the sample in a refrigerator at 4 °C. After these samples were washed twice with centrifugation for 3 h to remove excess lipases in the supernatant solutions, the immobilization of lipases inside the peptide nanotubes was confirmed by transmission electron microscopy (TEM, JEOL 1200EX) at 100 kV acceleration voltage. The TEM samples were prepared by dropping 10 μ L of the sample solution on carbon membrane 300 mesh copper grids and then the samples were dried completely in air. The loaded amount of lipase inside the nanotubes was determined by the colorimetric protein assay with a Bio-Rad reagent. After lipases and peptide nanotubes in the solution were separated by centrifugation, the lipase concentration in the supernatant was determined by the assay. The amount of lipases bound in the nanotubes was calculated by subtracting the lipase concentration in the supernatant from the initial lipase concentration. In this measurement, bovine serum albumin was applied as a standard. The resulting nanotubes, whose lipase loadings were determined by the assay, were used to study their activities and thermal stabilities after washing these nanotubes. To study the activities of those peptide nanotubes, 30 μ L of *p*-nitrophenyl butyrate solution (13.8 mg/mL in 2-propanol) was added to 200 μ L of the lipase–nanotube solution, and the mixture was balanced to 1 mL by a pH 7.4 buffer solution. The hydrolysis rates of *p*-nitrophenyl butyrate solutions were monitored by the product concentration, [*p*-nitrophenol], by means of UV–vis spectroscopy (Varian Cary 300 Bio). These processes are summarized in Figure 1. The thermal stability of lipases was also investigated at various reaction temperatures from 25 °C to 75 °C. In this thermal experiment, 6 μ L of lipase solution was mixed with 200 μ L of the nanotube solution at 4 °C for one week. After the resulting solution was diluted to 1 mL by a pH 7.4 buffer solution, the thermal stability of lipases were examined. Fluorescence studies of free-standing lipases and nanotube-bound lipases were carried out with a Jobin Ybin-SPEX, FL3-11 fluorometer. The excitation wavelength was 310 nm, and the bandwidth was 3 nm for both excitation and emission. This protocol allowed us to observe the tryptophan peak shift around 339 nm.

RESULTS AND DISCUSSION

A model enzyme, *Candida rugosa* lipase, was encapsulated within peptide nanotubes and its catalytic activity in hydrolysis of *p*-nitrophenyl butyrate was examined, as shown in Figure 1. The peptide nanotube was synthesized with the previously published method (18, 19). We used the peptide nanotube with the inner diameter of 160 nm, large enough to introduce substrates and lipases inside the nanotube while the ideal porous diameter of nanotube for lipase incorporation is not completely investigated yet.

After lipases were incubated in the nanotube solutions for one week, the inner wall of the nanotube was coated by lipases, as shown in Figure 2. Figure 2a shows TEM image of the peptide nanotube without incorporating lipases. Figure 2b is TEM image of the nanotube with 0.002 mg/mL of lipases. As shown in this TEM image, lipases appeared as darker areas in the nanotube because they were crystallized inside when the samples were dried on TEM grids (20). Figure 2c is TEM image of the lipase nanotube with 0.006 mg/mL of lipases. This TEM image shows that the inner wall of nanotube was fully coated by lipases. When the lipase concentration exceeded 0.009 mg/mL, the lipases were coated outside the nanotubes because the saturation of lipase binding on the inner wall induced the outside coating with the excess lipases. While we do not have direct evidence to explain this inside-coating mechanism, various nanoparticles were observed to coat inside the peptide nanotubes at lower nanoparticle concentrations via capillary effect (16, 21) and the trend of lipase incorporation at the lower concentrations is consistent with these previous outcomes. Another possible mechanism is the hydrophobic interaction between the nanotubes and the lipases. In general, the hydrophobic nonpolar residues of lipase spread on the outer shell while the enzymatic active sites are clustered (9, 22, 23), and then lipases aggregate on hydrophobic surfaces (24, 25). Therefore, the incorporation of lipases in the peptide nanotubes may also be due to the hydrophobicity of the inner surface of the peptide nanotube.

Enzymatic activities of lipases immobilized inside the peptide nanotubes were studied by monitoring the hydrolysis rate of *p*-nitrophenyl butyrate via the concentration change of a product, *p*-nitrophenol (Figure 1). To obtain their activities, the initial slopes of *p*-nitrophenol concentration changes over the reaction time were used. Figure 3 shows that the hydrolysis rate of lipases inside the peptide nanotubes increased linearly as a function of the lipase concentration. This feature indicates that the peptide nanotube support could maintain the enzymatic function of lipase, and the binding inside the nanotube did not hinder the catalytic reaction (26). Since the peptide nanotube itself has amino groups that may react with the resulting esters to affect the observed hydrolysis rates, the extent of the *p*-nitrophenyl ester hydrolysis by the neat peptide nanotubes without the enzyme was examined. This control experiment showed that the product concentration was 10^3 times lower as compared to the one by the peptide nanotubes with the enzyme, which means that the peptide nanotube itself cannot hydrolyze *p*-nitrophenyl ester efficiently. Since the product formation by the neat peptide nanotubes was negligible, the observed hydrolysis rates in Figure 3 are solely contributed by the lipases in the nanotubes.

The thermal stabilities of lipases (0.006 mg/mL) inside the nanotubes and free-standing lipases were compared in the temperature range, 25 °C to 75 °C, as shown in Figure 4. In this figure, the activity of the lipase was obtained by its initial slope of the *p*-nitrophenol concentration change over the reaction time, and the lipase activities were normalized by the hydrolysis rate of freestanding lipase at 22 °C. In the temperature range of 25–50 °C, the yield of *p*-nitrophenol by the nanotube-bound lipases is 33% higher than the one by the free-standing lipases. In the higher temperature range, the nanotube-bound lipases still maintained its activity up to 65 °C (a solid line in Figure 4); however the free-standing lipases lost their activity rapidly (a dotted line in Figure 4). As a result, the activity of nanotube-bound lipases was observed to be 70% higher as compared to the activity of free-standing lipases at 65 °C. The similar magnitude of the activity enhancement of nanotube-bound lipases was still observable at 75 °C. The drop of the activity for the lipases in the nanotubes at 75 °C (Figure 4) was not caused by the destruction of the peptide nanotube because the nanotube structure was observed to be rigid up to 200 °C (18). It is also unlikely that the activity decrease was caused by the lipase desorption since no lipases were released from the nanotubes in all temperature range during the activity measurement.

This outcome indicates that the noncovalent binding between the inner nanotube surfaces and lipases is strong enough to hold lipases during the enzymatic reaction even in the elevated temperatures. The increase of lipase activity is also likely contributed by the conformation change of lipases, which exposes the catalytic active sites of lipases as lipase molecules are attached on the peptide nanotubes. For example, when lipases were attached on an oil–water interface, their catalytic activity was enhanced dramatically because lipases could undergo the conformation change from a closed form (i.e., enzymatically inactive) to a more open form (i.e., enzymatically active) (27). This type of conformation change has been detected by monitoring intrinsic fluorescence intensity of tryptophan in lipases (28, 29). When lipases were unfolded to the open conformation, the hydrophobic pockets of lipases were exposed, which increased the fluorescence intensity of tryptophan. If the activity of lipases on the peptide nanotubes is increased with the same mechanism via the conformation change induced by the nanotube–water interface, the increase of the Trp fluorescence intensity of nanotube-bound lipases should be observed as compared to free-standing lipases. Figure 5 shows the fluorescence intensity change of lipases before and after adding the peptide nanotube solution. The exact peak positions of these spectra was determined by the Lorentzian fits, as shown in dotted lines in Figure 5. Indeed, the increase of the tryptophan fluorescence intensity at 339 nm (blue) was observed after lipases bound the nanotubes, which is consistent with the enhancement mechanism observed in the lipase system at the oil–water interface. The red-shift of the tryptophan peak from 333 nm (a peak of the red line in Figure 5) to 339 nm (a peak of the blue line in Figure 5) after the nanotube solution was added to lipases, is also consistent with the red-shift of the tryptophan's fluorescence spectra in polar environments. When we added the substrate to a solution of the peptide nanotubes without lipase and measured the amount of substrate remaining in solution by UV–vis absorption, the resulting substrate concentration was identical to the initial concentration of the substrate. Since there is no partitioning of substrates in the nanotubes over 24 h, the observed enhancement of lipase activity in the peptide nanotubes is not due to simply local increase of the substrate concentration inside the nanotube. The

conformation change of lipases may also indicate that the inner surface of the peptide nanotube facilitates lipases on the nanotube surfaces in the favorable orientation to maximize the contact with the substrate, *p*-nitrophenyl butyrate (9, 23).

In conclusion, peptide nanotubes were applied as supports for enzymes. We encapsulated a model enzyme inside peptide nanotubes, and the catalytic activity of nanotube-bound lipases was 33% higher as compared to free-standing lipases at room temperature. It was remarkable that the peptide nanotubes behaved as even better enzyme supports at higher temperature. At the elevated temperature, 65 °C, the activity of lipases inside the nanotubes was 70% higher as compared to free-standing lipases. The activity enhancement of lipases in the peptide nanotubes is likely induced by the conformation change of lipases to the open form (the enzymatically active structure) as lipases are adsorbed on the inner surfaces of peptide nanotubes. The stable binding between the nanotubes and lipases may also contribute to maintain the open (enzymatically active) form of lipases, which keeps the high catalytic activity at elevated temperature. It is advantageous to apply these peptide nanotubes as enzyme supports because the nanotubes can immobilize enzymes on the surfaces with a simple procedure and provide an appropriate environment for the active conformation of lipases (15, 17). The peptide nanotube can also be decorated by magnetic materials, which is beneficial to develop recyclable sensors and high-throughput catalysts (21).

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Figure 1.
Illustration of lipase nanotube fabrication and its enzymatic application.

Figure 2. TEM images of peptide nanotubes incorporating *Candida rugosa* lipase at (a) [lipase] = 0 mg/mL (b) [lipase] = 0.002 mg/mL (c) [lipase] = 0.006 mg/mL. Scale bar = 200 nm.

Figure 3. Hydrolysis rate of lipid catalyzed by lipases inside the nanotubes. A dotted line is a fit for experimental data points.

Figure 4. Thermal stability of lipases. The solid line and ■ represent lipases inside the nanotubes and the dotted line and ○ represent free-standing lipases.

Figure 5.

Fluorescence spectra of lipases bound the inside wall of peptide nanotubes (a blue line) and free-standing lipases (a red line). Dotted lines are the Lorentzian fits for these spectra and the computed peak positions by these fittings are also marked in this plot.