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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Stable and continuous long-term enzymatic reaction using an enzyme–nanofiber composite

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Abstract This study shows the preparation and application of enzyme–nanofiber composites for long-term stable operation. The enzyme–nanofiber composite was prepared by coating an enzyme aggregate, the esterase from *Rhizopus oryzae*, on the surface of the nanofibers. After immobilization on the nanofiber, the apparent K_m for the immobilized esterase was 1.48-fold higher than that of the free esterase, with values of 0.98 and 1.35 mM for the free and immobilized enzymes, respectively. It was found that enzyme–nanofiber was very stable, even when the fibers were shaken in glass vials, preserving 80% of the initial activity for 100 days. In addition, the enzyme–nanofiber composite was used repeatedly in 30 cycles of substrate hydrolysis and still remained active. Consequently, the esterase–nanofiber composite was employed within a continuous reactor system to evaluate its use in a long-term and stable continuous substrate hydrolysis reaction. It was found that the production of *p*-nitrophenol was stable for at least 400 h. This study demonstrates that the enzyme–nanofiber composite can be used in both repeated-batch mode and a continuous mode for a long-term stable operation.

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Introduction

Enzymes are powerful catalysts that are applied within various fields because they are highly specific and active. They are used in practical applications within various industries and fields, including biodegradation, biosensors, food processing, and the development of biofuel cells. Although a lot of merits exist in using enzymes in different protocols, their use is limited by their short active lifetimes. In general, enzymes become unstable over time due to changes in the vital three-dimensional structure. In addition, although enzymes can theoretically be reused for further catalysis with fresh substrate, the reuse of enzymes is difficult to achieve because it is not easy to separate the enzyme from reaction by-products.

To overcome these disadvantages, many researchers use immobilization techniques (Fernandez-Lorente et al. 2006;

Morana et al. 2006; Tardioli et al. 2006). The immobilization of enzymes allows their reuse as well as an extended life of activity. Conceptually, three ways are used to immobilize enzymes, i.e., adsorption, covalent binding, and encapsulation. With each of these, the carriers or matrices should be considered because they have an effect on the stability of the immobilized enzyme.

With recent progress in nanoscience, many new nanostructures are available for enzyme immobilization applications (Jia et al. 2002; Kim and Grate 2003; Lee et al. 2004; Sawicka et al. 2005; Wei et al. 2000). For instance, the use of nonporous materials enhanced biocatalytic efficiencies by minimizing diffusion limitations, whereas porous materials increased the amount of enzyme loading. Typically, smaller particles provide a larger surface area for the attachment of enzymes (Jia et al. 2003) and a shorter diffusional path for the substrates. Thus, nanoparticles were utilized as carriers for enzyme immobilization in many studies (Govardhan 1999; Haring and Schreier 1999; Xu and Klibanov 1996). Mesoporous silica, which has a well-ordered pore structure, has also garnered attention as an excellent support for enzyme immobilization. Because enzyme immobilization is dependent on the enzyme size (Diaz and Balkus 1996), many types of mesoporous materials that have different pore sizes, including MCM-41, SBA-15, and MCF, were developed for hosting enzymes (Schmidt-Winkel et al. 1999; Zhao et al. 1998).

Nanofibers have large surface areas because they are produced in a small string-like structure. A supporting scaffold with a large surface-to-volume ratio is desirable because a high catalytic efficiency can be achieved (Kim et al. 2005a, 2006). As such, it was assumed that nanofibers, with their high surface-to-volume ratios, would be good scaffolds for enzyme immobilization, whereas substrate diffusion limitations, an inherent problem when enzymes are trapped within nanopore-structured materials, are not as likely because the enzymes are located on the surface of the scaffolding (Kim et al. 2005b). Furthermore, enzyme–nanofiber composites can be recovered easily and used repeatedly. It was also reported that an enzyme aggregate coating on nanofibers improves the enzyme activity and stability (Kim et al. 2005a). The α-chymotrypsin-coated nanofibers, formed via cross-linking and aggregation, showed negligible loss of the enzyme activity for more than 1 month.

Because enzymes differ from one another in various characteristics, such as the functional groups present on their surface, their hydrophobicity, and their structure, it is not expected that a previously reported protocol for enzyme immobilization will be successful with every enzyme. As such, a protocol needs to be tested with each new enzyme. Likewise, the aim of various carrier-bound immobilized enzymes is to facilitate their use in continuous processes and, particularly, to overcome high costs by facilitating efficient separation, recycling, and reuse of the costly enzymes and the trouble-free control of the process. At present, most continuous enzymatic reactors that have been reported on are membrane reactors, i.e., they couple the membrane system with a continuous stirred tank reactor (CSTR; Gaouar et al. 1997; Jeon and Kim 2000; Kuo et al. 2004; Lin et al. 1997; Nidetzky et al. 1996; Satory et al. 1997).

In contrast, this study evaluated the use of nanofibers as the scaffolding for immobilization. Using a protocol modified from previous studies (Fischback et al. 2006; Kim et al. 2005a), we found that it is possible to very stably immobilize an esterase from *Rhizopus oryzae* on the nanofibers. Because esterases are able to catalyze the hydrolysis of esters into the corresponding carboxylic acids and alcohols and are broadly used in stereoselective hydrolysis and transesterification, the generation of a stably immobilized esterase is potentially important for pharmaceutical, food, biochemical, and biodegradation processes. Furthermore, this composite of the enzyme and nanofibers was successfully applied within a CSTR, demonstrating that this system can be used within a continuous operation, and potentially industrial applications. To our knowledge, no study has evaluated the applicability of nanostructure-immobilized enzymes for a continuous operation.

Materials and methods

Enzyme immobilization and materials

We used an esterase from *R. oryzae* to construct the enzyme–nanofiber composite. This enzyme was purchased from Biochemik Chemicals. The chemicals used, 4-nitrophenyl butyrate, glutaraldehyde, ammonium sulfate, and *N*,*N*-dimethylformamide, were purchased from Sigma-Aldrich (USA).

The nanofiber used in this study is composed of polystyrene (PS) and poly(styrene-*co*-maleic anhydride) (PSMA), and its preparation was described in a previous study (Kim et al. 2005a). In brief, a mixture of PS and PSMA with a 2:1 weight ratio was prepared in tetrahydrofuran and dissolved by stirring. The polymer mixture solution was loaded into a plastic syringe (Becton-Dickinson, Franklin Lakes, NJ, USA) equipped with a 30-gauge stainless steel needle (Precision-glide, Becton-Dickinson). A bias of 7 kV was applied to the needle using a high-voltage supply (ES30P-10W, Gamma High Voltage Research, Ormond Beach, FL, USA). The solution was fed at a rate of 0.15 ml/h using a syringe pump (PHD-2000 Infusion, Harvard Apparatus, Holliston, MA, USA). The electrospun fibers were collected on clean aluminum foil (connected to the ground and a high-voltage supply) placed at a suitable distance (7 cm) from the tip of the needle. The nanofibers produced were long strings with a 200- to 1,000-nm diameter.

To construct a stable esterase–nanofiber composite, we used a modified protocol based on previous studies (Fischback et al. 2006; Kim et al. 2005a). Initially, we prepared 10 mg/ml enzyme solutions in 100-mM phosphate buffer (pH 8.0). The nanofibers were incubated in glass vials containing 1 ml of the enzyme solution, and, after vortexing, the glass vials were shaken at 200 rpm at room temperature for 30 min. The glass vials were then moved to a rocker and shaken at 60 rpm at 4°C for 2 h. To form the enzyme aggregate, 1 ml containing 3% glutaraldehyde and 0.5-g ammonium sulfate were added to the vials, and these were placed in a shaker set at 200 rpm and at room temperature for 2 h, followed by an overnight incubation on a rocker (30 rpm) at 4°C. After transferring the enzyme–nanofiber composites to new glass vials, they were briefly washed with 100-mM phosphate buffer, and unbound functional groups on glutaraldehyde were capped using 100 mM Tris–HCl (pH 7.8). After capping, the enzyme–nanofiber composites were washed with 100-mM phosphate buffer (pH 6.5) until no enzyme was observed in the washing solution. The enzyme–nanofiber composites were then stored at 4°C with 1.5 ml of 100-mM phosphate buffer (pH 6.5) until required.

Activity measurement and stability

The activity of the immobilized esterase was measured using the production of *p*-nitrophenol from the hydrolysis of *p*-nitrophenyl butyrate dissolved in *N*,*N*-dimethylformamide. For activity measurements, the buffer in the vials was initially removed. The vials were refilled with 1.98 ml of 100-mM phosphate buffer (pH 6.5) and 20 μl of 50-mM substrate. After adding substrate, the vials were briefly vortexed and shaken at 200 rpm. The activity was calculated from the slope of $A_{400\ \mathrm{nm}}$ vs time, and the absorbance was equated with the concentration of *p*-nitrophenol. For stability measurements, the vials containing enzyme–nanofiber composites in 100-mM phosphate buffer (pH 6.5) were stored with shaking (200 rpm) at room temperature. At each time point, we measured the activity as described above. After measuring the activity, the enzyme–nanofiber composites were washed with 100-mM phosphate buffer (pH 6.5) five times for next measurement.

The same experimental protocol was used when we measured the stability of the enzyme during repeated use. Initially, 50 mM of *p*-nitrophenyl butyrate dissolved in *N*,*N*-dimethylformamide was prepared. The vials containing the esterase–nanofiber composites were filled with 20 μl of the substrate and 1.98 ml of 100-mM phosphate buffer (pH 6.5). The absorbance was measured at 400 nm, and from these values, the activities were calculated. Afterwards, the esterase–nanofibers were washed with 100-mM phosphate buffer and stored at room temperature in 2 ml of fresh phosphate buffer until the next measurement was performed.

One unit (U) of esterase activity is defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per minute under the assay conditions.

Continuous enzymatic reactor system

The schematic of the continuous reactor system is shown in Fig. 1. Basically, the continuous enzymatic reactor system is composed of two reactors, both having a working volume of 10 ml. One reactor contained the enzyme–nanofiber composite and is the working reactor, and the other is the control reactor, i.e., without the enzyme–nanofiber composite. Two tubes were connected to each reactor for inlet and outlet flows, respectively. Substrate was supplied to both reactors from a single reservoir, and the dilution rate was controlled by changing the flow rate. The continuous reactor system was operated in a cold room (4°C) to help stabilize the substrate in the reservoir solution during the long-term operation. However, the reactors have a water jacket, through which water from a water bath was run so that the temperature within the reactor was kept at 30°C. The reservoir solution was prepared by adding 50-mM 4-nitrophenyl butyrate (dissolved in *N*,*N*-dimethylformamide) to 100-mM phosphate buffer (pH 6.5) to a final concentration of 0.5 mM. The phosphate buffer and reactor system were autoclaved. The reactors contained magnetic bars and were placed on a magnetic stirrer during the operation to ensure that the solution was homogeneous. Initially, fresh phosphate buffer was supplied to both reactors until a sufficient flow was generated and then this was replaced with the buffer containing the substrate. Washing of the nanofibers was not a concern because they formed a tightly wound bundle that floated just beneath the surface of the solution. Meanwhile, the outlet tube was situated as close to the bottom of the reactor as possible to prevent plugging from occurring. At each time point, samples for the analysis of *p*-nitrophenol production were collected for 10 min from the outlet tubes, and 80 μl of the collection was diluted tenfold with fresh phosphate buffer (720 μl), and this was used to measure the absorbance at 400 nm. During sampling, the glass vials used to collect the samples were wrapped in aluminum foil and placed on ice to prevent the substrate from degrading. Because the *p*-nitrophenyl butyrate in the reservoir partially hydrolyzed during the experiments, the production of *p*-nitrophenol was determined using the differences in absorbance values seen between the sample and control reactors.

Data analysis

All samples were performed in triplicate for error analysis. The standard deviations for the results are shown as error bars within the graphs.

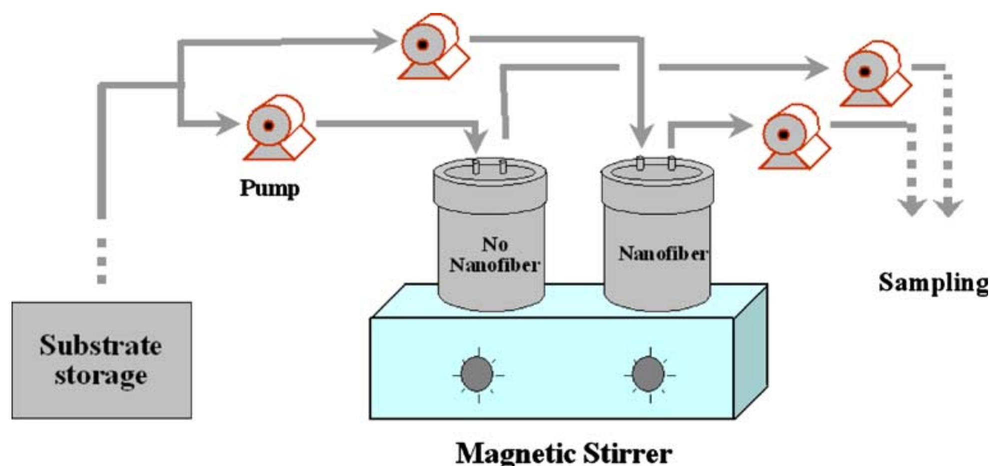

Fig. 1 Schematic of the continuous enzymatic reactor system

Results

Construction of esterase–nanofiber composite

We constructed enzyme aggregates on nanofiber through a modified protocol based on previously published works (Fischback et al. 2006; Kim et al. 2005a). After immobilization, 2 mg of the biocatalytic nanofiber showed an enzymatic activity of 2.6 mU. The Lineweaver–Burke plot for the free and immobilized enzymes gave estimated K_m values of 0.98 and 1.35 mM, respectively (Fig. 2). The apparent K_m for the immobilized esterase is 1.48-fold higher than that of free esterase.

Stability test

Enzyme stabilization is one of the main purposes that researchers use enzyme immobilization. To verify the stability of the esterase–nanofiber composite, we stored the esterase–nanofiber composite on a rotary shaker set at 200 rpm and at room temperature. For comparison, free enzyme was also stored under the same conditions. The stability was monitored for more than 100 days, and the results are shown in Fig. 3. The activity of the free esterase rapidly decreased, showing less than 10% residual activity after only 4 days. In contrast, the esterase–nanofiber sample initially had a decrease in its activity, but this leveled out at about 80% the initial value, and this activity was then maintained for more than 100 days. This is due to the fact that strong enzyme aggregates prevent the enzyme from leaching into solution and denaturing (Kim et al. 2005a). Furthermore, the activity of the enzyme–nanofiber composite was measured repeatedly, more than 30 times over the extent of this experiment, and was still active even after all of these measurements, preserving 82% of the initial activity.

Continuous enzymatic reactions using an esterase–nanofiber composite

The esterase–nanofiber composite was employed in a continuous enzymatic reactor to show the feasibility of long-term stable operation. The esterase–nanofiber composite in the reactor was suspended in the solution, which was continuously stirred using a magnetic stirrer. In this system, substrate was continuously supplied into the

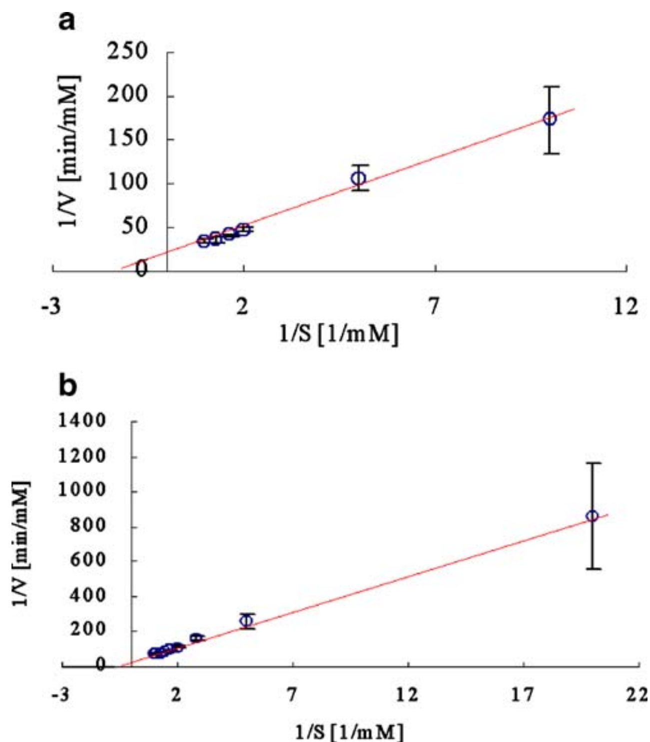

Fig. 2 Lineweaver–Burke plot for the free esterase (**a**) and the esterase-nanofiber composite (**b**). Each *symbol* and the *bars* represent the average value and the standard deviations, respectively. Results are from three samples taken at each time point

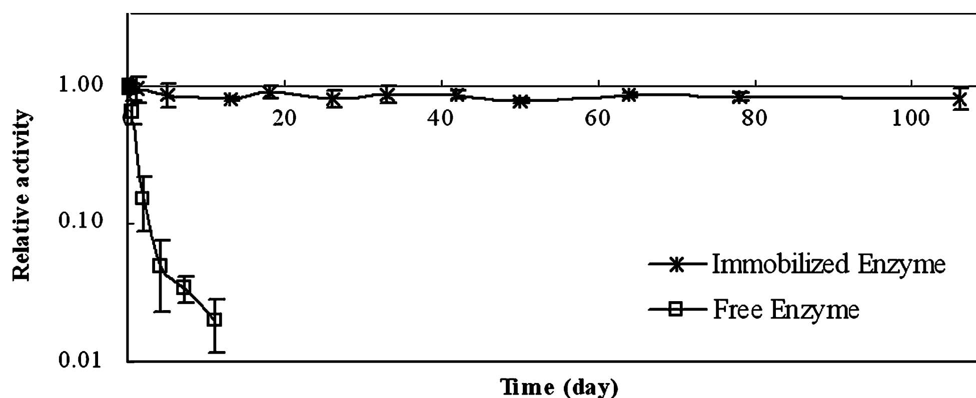

Fig. 3 Comparison of the stabilities of the immobilized and free esterases. The relative activity was calculated using the residual activity at each time point relative to that found initially on day 0

reactor, with a working volume of 10 ml, at different flow rates to change dilution rate.

During operation of the reactors, and for all the dilution rates tested, the production of *p*-nitrophenol followed a pattern similar to that seen in Fig. 4, i.e., with an initial increase in the *p*-nitrophenol concentration shortly after supplying the substrate to the reactor followed by a steady-state level. After reaching this steady-state level, the esterase–nanofiber composite worked stably in the reactor, as shown in the representative results in Fig. 4. The esterase–nanofiber composite did not lose any apparent activity during the operation of the reactor, whereas the capability of the esterase–nanofiber composite was constant during long-term operation, which indicates that this esterase–nanofiber composite is stable, even in a continuous reactor system. As shown in Fig. 4, this system maintained a steady-state production level for 400 h with a dilution rate of 0.3 h^{-1}, and to our knowledge, no study shows a similar long-term stable operation of a continuous enzymatic reactor.

As expected, it was found that the amount of *p*-nitrophenol produced can be controlled by modifying the dilution rate. For instance, with a dilution rate of 0.1 h^{-1}, the degree of hydrolysis was 1.8 times higher than that at 0.6 h^{-1} (data now shown).

Discussion

After immobilization on the nanofiber, only a small change in the K_m value for the esterase was seen when compared to the free enzyme. This result demonstrates that the affinity of the enzyme for the substrate has not changed significantly and that the binding domain in the enzyme is not significantly affected due to its immobilization on the nanofibers. One possible reason for the difference seen is that there would be a reduced diffusion path for the substrate around the nanofiber.

One benefit found from the nanofiber-immobilized enzyme system is the enhanced stability of the esterase. In experiments with both a batch reactor system (flask) and a CSTR, denaturation of the protein and the subsequent loss of activity were not a concern, even after numerous repeated usages or an extended continuous use, as in the case of the CSTR. Stability of the enzyme is important in these processes because the

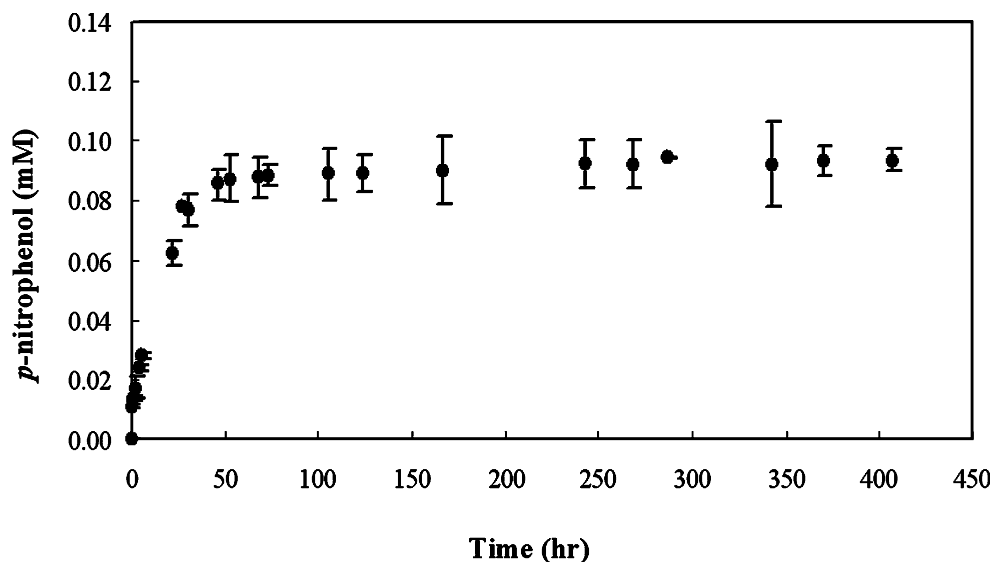

Fig. 4 *p*-nitrophenol formed due to enzymatic reaction during long-term operation of the continuous enzymatic reactor, showing the stability of the system. D=0.3 h^{-1}. The concentrations of *p*-nitrophenol were obtained using the difference in the absorbance between the sample and control reactor reading

productivity of the reaction is dependent upon this activity and enzymes tend to be expensive and/or require a lengthy process to purify, even from recombinant sources. This benefit is further enhanced by the fact that the morphology of the nanofibers can easily be changed to form mats, arrays, and membranes (Kim et al. 2007). Thus, similar enzyme–nanofiber composites can be applied within continuous reactor systems using a variety of forms that fit the experimental scheme desired. Therefore, the enzyme–nanofiber composites can be used in different types of reactors, such as batch, plug flow, and continuous reactors, as well as other types of systems, to achieve an optimum productivity or to satisfy the purpose of the processes. Furthermore, in situations where the activity of the enzyme–nanofiber composites within the reactor is stable, as was seen in this study, the concentration of the end products can be optimized by simply modifying the substrate concentration and/or the dilution rate.

Conclusions

This study demonstrated that the enzyme–nanofiber composite was stable in its substrate hydrolysis reaction in both repeated-batch and continuous long-term operation modes. Initially, a stable esterase–nanofiber composite was constructed using an esterase from *R. oryzae*. After immobilization, the K_m value of the enzyme was not significantly different from that of the free condition enzyme. However, the immobilized esterase was significantly more stable, showing little loss in activity even when on a shaking incubator for more than 100 days. Furthermore, it can be reused. Experiments showed that the esterase–nanofiber composite kept 82% of the initial activity level after 30 hydrolysis experiments.

An enzymatic reaction employing the esterase–nanofiber composite was found to be very successful in its long-term continuous substrate hydrolysis. Shortly after addition of the substrate to the reactor, the process reached a steady-state *p*-nitrophenol production level that was consistent throughout the life of the experiment. During a long-term operation, the reactor system was continuously and stably operated for more than 400 h, demonstrating that the enzyme–nanofiber is applicable within a continuous system.

Taking into consideration the various applications in which enzymes are currently used, enzyme–nanofiber composites provide numerous advantages to various fields of study and development, including food, pharmaceutical, and bioremediation processes, all of which involve the use of costly enzymes.

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