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Design and synthesis of lipase nanogel with interpenetrating polymer networks for enhanced catalysis: Molecular simulation and experimental validation

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ABSTRACT

A temperature-responsive lipase nanogel (denoted as CRL-IPN nanogel), in which lipase is encapsulated into an interpenetrating polymer matrix formed by polyacrylamide and poly(*N*-isopropylacrylamide) (PNIPAAm) has been designed and synthesized for an enhanced stability and activity in both aqueous and non-polar organic solvents. A three-step method, including acryloylation, polymerization with acrylamide and sequential polymerization with *N*-isopropylacrylamide, was established to fabricate enzyme nanogel with temperature-sensitive interpenetrating polymer network. It has been shown by an all-atom molecular dynamics simulation that above mentioned polymer matrix forms a more hydrophobic environment, as compared to that obtained with sole polyacrylamide, because of the penetration of *N*-isopropylacrylamide into the polymer acrylamide network *via* hydrogen bonding, which is further confirmed by the fluorescence spectrum. This favours the uptake of hydrophobic substrates and thus the overall rate of enzymatic catalysis. The enhanced stability and catalytic performance of this novel lipase nanogel in aqueous and non-polar organic solvent were demonstrated by using hydrolysis reaction of *p*-NPP in aqueous and esterification reaction of ibuprofen in isooctane. In aqueous solution, the residual activity of CRL-IPN nanogel maintains its 70% activity at 60 °C after 4 h, compared with that free lipase only has 30% at the same condition. In addition, the CRL-IPN nanogel can be reused for 10 cycles with no loss of its activity. In isooctane, CRL-IPN nanogel gave a 33% yield of esterification of ibuprofen, in comparison to 22% using free lipase and less than 5% using lipase encapsulated in a polyacrylamide matrix. The enhanced stability and activity make this CRL-IPN nanogel promising for enzymatic catalysis in non-polar solvents.

1. Introduction

Biocatalysts hold great promise for chemical synthesis due to their high efficiency, high selectivity, and environmental friendliness [1–3]. The limited stability of biocatalysts in industrial environments, however, significantly hinders their applications [2]. In general, there are two ways to enhance protein stability under harsh conditions. One is based on protein engineering such as directed evolution and site-specific mutation [4,5]. The other is through chemical modification [6–11], which can impart more diversity in terms of protein structure and function due to extensive chemical libraries [12]. The incorporation of enzymes into nanostructured materials is particularly noteworthy from a structural perspective for its capability of tailoring the environment for chosen enzymes [13,14]. To date, the established methods have been grouped into three major categories, namely "grafting onto", "grafting from", and "self-assembly", according to the synthetic procedures involved [14]. It has been proven that nanostructured enzymes offer advantages in terms of high surface area, enhanced stability under adverse conditions, and enriched structural compatibility, with only a marginal increase in mass-transfer resistances. These features essentially enable an extended application spectrum of enzymatic catalysis [15–20].

Aqueous synthesis of enzyme nanogels by two-step *in situ* polymerization has proven to be an effective means of fabricating nanostructured enzyme catalysts with enhanced stability at high temperature and in the presence of organic solvents [21–24]. Acrylamide is by far the most widely applied monomer, which forms a hydrophilic nanogel matrix that contributes to the enhanced stability against high temperatures and denaturing solvents such as DMSO [25]. Unfortunately, however, this hydrophilic polymer matrix does not favour enzymatic catalysis in non-polar solvents, in which many valuable reactions proceed. It is well established that water is essential to both stability and catalytic behaviour of enzyme [26]. The hydrophobic channel, which favours transport of hydrophobic substrates, is essential for biocatalysis occurred

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in non-polar organic solvent. Therefore, construction of enzyme nanogel with both hydrophobic channel and water accessibility is crucial for biocatalyst with high performance. Another important problem is the recycle of enzyme nanogel. The extreme small size makes enzyme nanogel difficult to be reused, which hinders its application in biocatalysis.

To solve the above-mentioned problem, we came to the idea of fabricating an interpenetrating polymer network, which is composed by polyacrylamide and poly(*N*-isopropylacrylamide). Polyacrylamide provides a hydrophilic environment, which keeps essential water and stabilizes the enzyme stability, while poly(*N*-isopropylacrylamide) contributes to an improved solubility of enzyme nanogel in non-polar solvents, which favours transport of hydrophobic substrates and endows the temperature responsive properties of enzyme nanogel to recycle. Temperature-sensitive hydrogels have enormous potential in various applications, especially in the field of drug delivery [27]. The target molecules, such as small molecule drugs or proteins, can freely enter into or escape from temperature-sensitive hydrogels under an environmental stimulus. In our study here, however, to ensure a high catalytic efficacy while facilitating the recycle of the enzyme catalyst, an IPN of nanometer in depth was grafted from the enzyme surface *via* acryloylation and sequential polymerization that cooperated the temperature responsive segment, PNIPAAm, into the polymer network.

This work started with the fabrication of lipase nanogel by different strategies, and eventually a lipase nanogel composed of interpenetrating polyacrylamide/poly(*N*-propylacrylamide) networks was successfully synthesized by a stepwise fabrication method. In order to explain the mechanism of fabrication of this interpenetrating network lipase nanogel, an all-atom molecular dynamics simulation has been performed to provide molecular insight into the assembling process of acrylamide and *N*-isopropylacrylamide monomers and mixtures thereof around lipase. The synthesized interpenetrating lipase nanogel was then used for the esterification of ibuprofen in non-polar organic solvents, a reaction that could not be accomplished with lipase-acrylamide nanogel. The temperature-sensitive nature of *N*-propylacrylamide was exploited for the recovery of the lipase nanogel formed with a polyacrylamide/poly(*N*-isopropylacrylamide) interpenetrating matrix. Finally, the mechanism underpinning the observed drastic differences in enzymatic catalytic behaviour in non-polar organic solvents is discussed on the basis of molecular simulations.

2. Experiments and simulations

2.1. Materials

Lipase from *Candida rugosa* (Type VII, L-1754) (CRL), *p*-nitrophenylpalmitate (*p*-NPP), acrylamide (AM), *N*-isopropylamide (NIPAM), and *N*-acryloxysuccinimide (NAS) were purchased from Sigma–Aldrich, U.S.A. Sephacryl™ high-resolution S-400HR for size-exclusion chromatography was purchased from GE Healthcare. Ammonium persulfate (APS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), *N*,*N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and other chemicals were obtained from standard local suppliers and, unless stated otherwise, were of analytical purity and were used without further purification.

2.2. Preparation of lipase solution

Candida rugosa (Type VII, sigma) lipase (30 mg) was dissolved in acetate buffer (5.0 mL; 50 mM, pH 4.0). After centrifugation for 10 min at 9827 × *g* (10,000 rpm), the precipitate was removed and the solution was dialyzed against phosphate buffer (50 mM, pH 7.0) overnight. The solution was then stored at 4 °C and used for subsequent experiments.

2.3. Synthesis of lipase nanogel

The synthetic procedure for obtaining lipase nanogel involves generally two steps, namely acryloylation and *in situ* polymerization. For the acryloylation, a solution of NAS (15.0 mg) in DMF (400 μL) was slowly added to 5.0 mL of lipase solution and the mixture was incubated at 30 °C with stirring for 2 h. The acryloylated lipase solution was then dialyzed against phosphate buffer (50 mM, pH 7.0) at 4 °C overnight. For the *in situ* polymerization, acryloylated lipase solution (5 mL, 2.0 mg/mL) containing acrylamide (250 mg) or *N*-isopropylacrylamide (250 mg) or a mixture thereof (1:1 molar ratio) was prepared in a vial and purged with nitrogen. Radical polymerization on the surface of the acryloylated lipase was initiated by adding ammonium persulfate (15.0 mg) and *N*,*N*,*N*,*N*-tetramethylethylenediamine (12 μL) to the vial. The reaction was allowed to proceed at 30 °C for 6 h under a nitrogen atmosphere. Finally, the solution was dialyzed against water for 24 h at 4 °C to remove unreacted monomer and initiator.

For the fabrication of the interpenetrated nanogel, a lipase-polyacrylamide nanogel solution (5 mL, 2.0 mg/mL lipase) containing *N*-isopropylacrylamide (250 mg) was prepared in a vial and purged with nitrogen. Radical polymerization was then initiated by addition of ammonium persulfate (15.0 mg) and *N*,*N*,*N*,*N*-tetramethylethylenediamine (12 μL) to the vial. The reaction was allowed to proceed at 30 °C for 6 h under a nitrogen atmosphere. Finally, the solution was dialyzed against water for 24 h at 4 °C to remove unreacted monomer and initiator.

2.4. Assays

Protein concentration assay: The protein concentrations in the solutions, both free and in the nanogel, were determined by a bicinchoninic acid (BCA) colorimetric protein assay.

Determination of reacted amine groups: The number of unreacted amine groups on the acryloylated lipase was determined by trinitrobenzenesulfonate colorimetric assay (TNBS). Briefly, 6 μL of 2,4,6-trinitrobenzenesulfonic acid at a concentration of 2.5% (w/v) was added to 1 mL of the protein sample dissolved in pH 9.6 100 mM boric acid buffer. After incubation in the dark for 2 h at 30 °C, the absorbance of the sample was measured at 420 nm. The number of amine groups on the free lipase was also determined and used as a control.

Transmission electron microscopy (TEM): TEM images of the samples at 100,000× magnification were acquired using a Hitachi H-7650B high-resolution transmission electron microscope. Standard grids were photographed to determine the magnification. During a run, the protein nanogel sample was diluted with water to give a concentration of 0.01 mg/mL. Carbon-coated grids were prepared by adding a drop of protein solution, removing the excess, and applying 1% sodium phosphotungstate solution (pH 7.0). The sample was then subjected to TEM measurement [28].

Size-exclusion chromatography (SEC): SEC was conducted using a Sephacryl™ high-resolution S-400HR column (GE Healthcare) with UV/Vis detector (SPD-10Avp, Shimadzu) and fluorescence detector (RF-10AxL, Shimadzu). The yield of encapsulated lipase was compared to the total peak area of protein fractions, based on the UV absorption at 280 nm.

Fluorescence spectroscopy: Fluorescence spectrometric detection was accomplished by means of an RF-5301 PC (Shimadzu), with an excitation wavelength of 285 nm and an emission wavelength in the range 300–550 nm.