Materials Science and Engineering C 38 (2014) 278–285

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Covalent immobilization of porcine pancreatic lipase on carboxyl-activated magnetic nanoparticles: Characterization and application for enzymatic inhibition assays

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ARTICLE INFO

Article history:
Received 24 October 2013
Received in revised form 6 January 2014
Accepted 8 February 2014
Available online 15 February 2014

Keywords:
Lipase
Immobilized enzymes
Bioreactors
Enzyme activity
Magnetic nanoparticles
Covalent cross-linking

ABSTRACT

Using carboxyl functionalized silica-coated magnetic nanoparticles (MNPs) as carrier, a novel immobilized porcine pancreatic lipase (PPL) was prepared through the 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) coupling reaction. Transmission electron microscopic images showed that the synthesized nanoparticles (Fe_3O_4–SiO_2) possessed three dimensional core–shell structures with an average diameter of ~20 nm. The effective enzyme immobilization onto the nanocomposite was confirmed by atomic force microscopic (AFM) analysis. Results from Fourier-transform infrared spectroscopy (FT-IR), Bradford protein assay, and thermo-gravimetric analysis (TGA) indicated that PPL was covalently attached to the surface of magnetic nanoparticles with a PPL immobilization yield of 50 mg enzyme/g MNPs. Vibrating sample magnetometer (VSM) analysis revealed that the MNPs-PPL nanocomposite had a high saturation magnetization of 42.25 emu·g^{-1}. The properties of the immobilized PPL were investigated in comparison with the free enzyme counterpart. Enzymatic activity, reusability, thermo-stability, and storage stability of the immobilized PPL were found significantly superior to those of the free one. The K_m and the V_{max} values (0.02 mM, 6.40 U·mg^{-1} enzyme) indicated the enhanced activity of the immobilized PPL compared to those of the free enzyme (0.29 mM, 3.16 U·mg^{-1} enzyme). Furthermore, at an elevated temperature of 70 °C, immobilized PPL retained 60% of its initial activity. The PPL-MNPs nanocomposite was applied in the enzyme inhibition assays using orlistat, and two natural products isolated from oolong tea (i.e., EGCG and EGC) as the test compounds.

1. Introduction

Lipase (triacylglycerol acyl hydrolase, EC 3.1.1.3) catalyzes the hydrolysis of triglycerides and other carboxylic esters into monoacyl glycerols and free fatty acid, and thus has been widely used in chemical and pharmaceutical industries [1]. In particular, since its inhibitors can adjust fat absorption, lipase has become an important target in drug discovery for searching potential anti-obesity compounds [2]. Lipase obtained from pig's pancreas (porcine pancreatic lipase, PPL) is the most extensively used one due to its convenient accessibility, high stability, and broad specificity in transesterification reactions [3]. PPL is a small globular protein composed of a single chain of 449 amino acids with molecular weight of 50–52 kDa, and because it possesses 86% of homology with human pancreatic lipases, it has been usually used as a substitution for human pancreatic lipase in the enzyme inhibitor screening [4,5].

In many enzymatic reactions, immobilization of enzymes may provide significant improvements from economical and technical points of view. Immobilized enzymes present several benefits over the free one such as enhanced stability, ease of separation from the reaction mixture, significant savings in enzyme consumption, and usually improved activity [6]. The immobilization methods include physical adsorption, covalent attachment and entrapment in inorganic and organic matrices [3]. Up to date, PPL has been immobilized with various solid supports including macroporous anion exchange resin, magnetic microspheres, chitosan beads, porous silica beads and polyvinyl alcohol [3,7,8]. The critical shortcoming of physical adsorption is that the immobilized enzymes are easily leached out from porous materials, resulting in enzyme loss and poor operational stability [9,10]. Covalent immobilization can bind enzymes to the solid supports stably, thus preventing leakage of the enzymes. As evidenced by many works, covalent bonding can either improve enzyme activity by stabilization of its active conformation, or affect the active centers of the enzymes to

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http://dx.doi.org/10.1016/j.msec.2014.02.011
0928-4931/

decrease their enzymatic activity. Therefore, development of appropriate covalent bonding methods is of vital importance for the immobilization of the enzymes.

Magnetic nanoparticles (MNPs) have gained popularity as immobilizing supports for bio-macromolecules because of their unique magnetic responsibility, low toxicity, and chemically modifiable surface, large enzyme loading capacity, and good reusability. Various enzymes have been immobilized on magnetic nanoparticle surfaces through a variety of functional groups including amine, aldehyde, carboxylic, epoxy, mercapto, and maleimide ends, and both enzymatic activities and stabilities were significantly improved [11]. In previous reports, amine and epoxy group functionalized silica-coated magnetic particles were used to covalently immobilize lipase [12,13]. Those results suggested that covalent immobilization of PPL on magnetic nanoparticles could result in higher enzymatic activity and stability.

In this work, covalent immobilization of PPL on carboxyl-terminated magnetic nanoparticles was carried out, and the PPL-functionalized nanocomposite (MNPs-PPL) was used for lipase inhibition assays. Various analytical techniques were used to characterize the enzyme modified nanocomposite in terms of optimal temperature and pH value for enzymatic activity, thermal stability, reusability and storage ability. Furthermore, the application of the immobilized PPL was extended to screening for lipase inhibitors with two natural products isolated from oolong tea (i.e., EGCG and EGC) and orlistat, a well-known lipase inhibitor. To date, the immobilized PPL was mainly limited to biocatalyzation in kinetic resolution of highly diverse racemic mixtures for the products of industry, ester synthesis and esterification conversion in the organic synthesis [14–17], and this is the first report that the magnetically separable biocatalysts be applied in the screening of lipase inhibitors.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), PPL (Type II, from porcine pancreas), *N*-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), orlistat (≥98%), 4-nitrophenol (*p*-NP), and 4-nitrophenyl palmitate (*p*-NPP) were purchased from Sigma-Aldrich Chemistry Co., Ltd. (United States). Tetraethyl orthosilicate (TEOS) and 3-aminopropyltrimethoxysilane (APTMS) were purchased from TCI (Tokyo, Japan). Succinic anhydride, gum arabic, Triton X-100 and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) were purchased from Tianjin Kermel Chemical Reagent Factory, China. $FeCl_3 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$, and *N*,*N*-dimethylformamide (DMF) were purchased from Chengdu Tianhua Chemical Technology Co., LTD. 2-(*N*-morpholino)-ethanesulfonic acid (MES) and Coomassie brilliant blue were purchased from Oumai Biotech Corporation (China). (−)-Epigallocatechin 3-*O*-gallate (EGCG) and (−)-epigallocatechin (EGC) (≥98%) were obtained from Chengdu Mansite Bio-tech Co. (Chengdu, China). Methanol and acetic acid used for HPLC were of chromatographic grade (JT Baker, USA). Purified water from a Milli-Q water system (Millipore Corp., Bedford, MA, USA) was used for sample preparation and analysis. The other chemicals and solvents were of analytical reagent grade.

2.2. Apparatus

HPLC analysis was performed on Shimadzu LC-20AD series HPLC system equipped with a Tianhe kromasil C_{18} column (250 × 4.6 mm, 5 μm particle) at constant temperature (35 °C) and an ultraviolet detector operated at 317 nm wavelength. The sample was separated with linear gradient elution of A: water and B: methanol, starting from 40% B to 80% B in 25 min. Flow rate was set at 0.8 $mL \cdot min^{-1}$. A UV-1800 spectrophotometer (Shimadzu Instruments, Inc., Tokyo, Japan) was used for sample absorbance measurements in the experiments. The sizes and morphologies of magnetic nanoparticles were tested using a transmission electron microscope (TEM, JEM-100CX, JEOL Co., Japan). AFM images were obtained with a Veeco Multimode AFM equipped with a Nanoscope IIIa controller. A RTESP silicon probe (Veeco, USA) was used (spring constant was 20–80 N/m, resonance frequency ranging from 235 to 287 kHz). AFM measurements were carried out in air at room temperature using tapping mode. For each sample, AFM scans (typically 1 × 1 μm) were carried out on several surface positions to check the surface uniformity. The magnetic properties of the prepared nanoparticles were measured on a vibrating sample magnetometer (VSM, Quantum Design Company, USA). Fourier-transform infrared spectra (FTIR) were obtained using a Perkin-Elmer FT-IR Spectroscopy (KBr). Thermo-gravimetric analysis (TGA) was performed for powdered samples with a heating rate of 10 $°C \cdot min^{-1}$ from room temperature to 700 °C under a high purity nitrogen atmosphere using a TGA Q500 V20.10 Build 36 thermo-analysis system (American TA Instruments Cot., USA). All pH measurements were made with a pHS-3 digital pH-meter (Shanghai LeiCi Device Works, Shanghai, China) with a combined glass–calomel electrode.

2.3. Preparation and characterization of magnetic nanoparticles covered with carboxyl groups

The magnetic Fe_3O_4 nanoparticles covered with carboxyl groups were synthesized as in the following steps. Firstly, the magnetic Fe_3O_4 nanoparticles (MNPs) were synthesized by co-precipitation of Fe^{2+} and Fe^{3+} with ammonia water as the precipitant [18]. Under vigorous stirring, ammonia water was dropped into the mixture solution of Fe^{2+} and Fe^{3+} (molar ratio = 2:1) till the pH value reached to about 10, and the stirring continued for 30 min to obtain black magnetite particles. Secondly, the black magnetite particles were covered with silica based on the Stöber method [19]: the black magnetite was ultrasonically dispersed into a mixture of ethanol–water solution, NH_4OH and TEOS, and the mixture was stirred for 6 h to produce the core–shell structured $Fe_3O_4@SiO_2$ which was collected with an external magnet and washed sequentially with water and ethanol. Thirdly, the $Fe_3O_4@SiO_2$ was redispersed into 150 mL ethanol/water (v:v, 1:1) solution, and stirred with 200 μL of APTMS under N_2 protection at 40 °C for 8 h to introduce amine groups on the surface of $Fe_3O_4@SiO_2$ [20]. Finally, the amino-modified particles $Fe_3O_4@SiO_2–NH_2$ were stirred with 10% succinic anhydride in DMF solution under nitrogen atmosphere for 3 h at room temperature, to obtain the final carboxyl-modified magnetic nanoparticles $Fe_3O_4@SiO_2–NH_2–COOH$, which after centrifugation and washing with water, were dried under vacuum at 40 °C [21].

2.4. PPL immobilization

Before the PPL immobilization, carboxyl groups in $Fe_3O_4@SiO_2–NH_2–COOH$ were activated using EDC/NHS according to the method previously reported [22]. In brief, EDC·HCl (5 mg) and NHS (7 mg) were dissolved in 3 mL of MES buffer (50 mM, pH 6.5), to which $Fe_3O_4@SiO_2–NH_2–COOH$ magnetic nanoparticles (20 mg) were added. The reaction was conducted at a water bath of 0 °C for 30 min to obtain an active ester. The MNPs with the active ester were collected by an external magnet and redispersed in 3 mL Tris–HCl buffer (50 mM, pH 6.5) containing free enzyme solution, and then shaken at room temperature for 24 h. The immobilization procedure of PPL on the MNPs is illustrated in Fig. 1. The immobilized PPL was magnetically separated and washed several times with Tris–HCl buffer (50 mM, pH 7.0) until no free enzyme was detected in the washing solution. The final immobilized PPL was redispersed in 1 mL buffer and stored at 4 °C before use. The washing solutions were combined for protein assay with Bradford assay using BSA as a standard. The immobilized amount of PPL on the magnetic nanospheres was calculated by subtracting the amount in washing solution from the total amount added in the immobilization process. The immobilization yield was defined as the amount of enzyme (mg) per gram of MNPs support.