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Fabrication of enzyme-immobilized halloysite nanotubes for affinity enrichment of lipase inhibitors from complex mixtures

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ABSTRACT

Lipase is the key enzyme for catalyzing triglyceride hydrolysis in vivo, and lipase inhibitors have been used in the management of obesity. We present the first report on the use of lipase-adsorbed halloysite nanotubes as an efficient medium for the selective enrichment of lipase inhibitors from natural products. A simple and rapid approach was proposed to fabricate lipase-adsorbed nanotubes through electrostatic interaction. Results showed that more than 85% lipase was adsorbed into nanotubes in 90 min, and approximately 80% of the catalytic activity was maintained compared with free lipase. The specificity and reproducibility of the proposed approach were validated by screening a known lipase inhibitor (i.e., orlistat) from a mixture that contains active and inactive compounds. Moreover, we applied this approach with high performance liquid chromatography–mass spectrometry technique to screen lipase inhibitors from the *Magnoliae cortex* extract, a medicinal plant used for treating obesity. Two novel biphenyl-type natural lipase inhibitors magnotriol A and magnaldehyde B were identified, and their IC_{50} values were determined as 213.03 and 96.96 μM, respectively. The ligand–enzyme interactions of magnaldehyde B were further investigated by molecular docking. Our findings proved that enzyme-adsorbed nanotube could be used as a feasible and selective affinity medium for the rapid screening of enzyme inhibitors from complex mixtures.

1. Introduction

Triglyceride hydrolysis is catalyzed by lipase in vivo, and this process can lead to the accumulation of free fatty acids and monoacylglycerol in the body. The re-absorption of these constituents by the intestinal system may induce obesity and other relevant diseases [1,2]. Lipase inhibitors, such as orlistat, which forms covalent binding to the enzyme (lipase) and hence inhibits the triglyceride hydrolysis [3,4], have become an efficient way to control or treat obesity [5,6]. Natural products are promising resources of lipase inhibitors. Various naturally derived lipase inhibitors have been reported in recent years and have been shown to have potentials for drug development [7–9]. However, the screening and identification of lipase inhibitors from natural products are time-consuming and labor-intensive works that often involve repeated bioassay guided isolation and identification [8,10]. Thus, the rapid screening of lipase inhibitor from complex mixture of natural products is of great demand to discover novel therapeutics for diseases related to abnormal fat accumulation.

During the last decade, various nanomaterials, such as carbon and TiO_2 nanotubes have been used as microextraction mediums for the selective enrichment of specific compounds [11,12]. Furthermore, a number of affinity mass spectrometry-based approaches, such as ultrafiltration [13–15], enzyme-immobilized magnetic particles, enzyme reactors [16–18], and cell membrane chromatography [19], have been developed to explore potential ligands from complex mixtures. Recently, we have demonstrated that enzyme-immobilized magnetic beads can be used for the screening of active compounds from herbal extracts [20]. Compared with commonly used immobilization techniques based on covalent attachment or cross-linking, the adsorption of enzymes onto the carriers offers advantages, including (1) the retention of active conformation of free enzyme and (2) avoidance of complicated pretreatment and chemical modification processes. Meanwhile, the physical/chemical property of the carriers (e.g. surface area) is critical to the efficacy of ligand screening and to the extent of non-specific binding, which may confound the interpretation of results.

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Halloysite, a naturally occurring aluminosilicate nanotube, has attracted increasing interest because of its outstanding physical characteristics. The inner diameter, external diameter and length of halloysite nanotubes (HNTs) are 20–30 nm, 30–50 nm, and 1–2 μm, respectively [21]. HNTs provide an ideal nanometer-scale entrapment system for the storage of drugs, enzymes, and biocides [22,23]. More importantly, the external (mainly composed of O—Si—O groups) and inner surfaces (consist of Al_2O_3) of halloysite nanotubes provide more selective enzyme binding and hence reduce the non-specific adsorption of ligands onto HNTs. Therefore, HNTs may be used as a novel medium for ligands screening.

The aim of this study is to develop a rapid and effective method to screen and characterize lipase inhibitors from *Magnoliae cortex* by lipase-adsorbed nanotube combined with HPLC–MS analysis. *Magnoliae cortex*, the bark of *Magnoliae officinalis* L. (Magnoliaceae), is a medicinal herb commonly used for digestive diseases. Many other biological activities have been associated with the extracts and ingredients of *Magnoliae cortex*, such as anti-inflammatory [24–28], anti-oxidant [29,30], anti-microbial [31], anti-clastogenic activities [32], and modulating the proliferation and migration of vascular smooth muscle cells [33]. To date, limited studies have been reported to identify the active compounds responsible for its efficacy in treating digestive diseases. In this study, four neolignan compounds were found to be potential ligands of lipase. Two of them were identified, and their structures were confirmed by nuclear magnetic resonance (NMR). Furthermore, their lipase inhibitory activities were verified by a functional assay. This new type of lipase inhibitors from *Magnoliae cortex* was reported for the first time.

2. Experimental

2.1. Materials and reagents

The halloysite nanotubes were obtained from Wenzhou Xincheng Shenfei Aluminum Alloy Co., Ltd., China. Cortex *M. officinalis* was obtained from the Hangzhou traditional Chinese herbal medicine factory (Hangzhou, China). Lipase (from porcine pancreas), 4-methylumbelliferyl oleate, orlistat and 4-nitrophenyl palmitate (pNPP), were purchased from Sigma-Aldrich Co., Ltd. (St. Louis, Missouri, USA). Schisandrin was purchased from Ronghe Pharmaceutical Technology Development Co. Ltd. (Shanghai, China). HPLC-grade methanol and acetonitrile were purchased from Merck. Formic acid (HPLC-grade) was purchased from Roe Scientific Inc. (Newark, DE, USA).

2.2. Characterization of lipase immobilized halloysite nanotubes

The morphological characteristics of the halloysite nanotubes and lipase-immobilized HNTs were obtained by transmission electron microscopy (TEM). Samples were prepared by drying drops of a diluted aqueous solution of empty HNTs or enzyme-immobilized HNTs onto a carbon-coated copper grid. TEM analysis was performed on a JEM-3010 TEM at 80 kV. The Fourier-transform infrared (FT-IR) spectra of these samples were recorded by a Thermo Nicolet Avatar 370 FT-IR spectrometer in transmission mode between 400 and 4000 cm^{-1} at a resolution of 4 cm^{-1} s.

2.3. Optimization of operative parameters for lipase immobilization

To improve the binding efficiency of lipase on HNTs, parameters including lipase concentration, HNTs-to-enzyme ratio, pH of Tris buffer, incubation time, and incubation temperature were optimized. Different amounts of HNTs (5, 10, 20, 40, 60, and 80 mg) were mixed with lipase (1 mL, 2 mg mL^{-1}) at various time intervals (30, 60, 90, 120, 180, and 240 min). After incubation at a specific time, the mixture was washed three times with 1 mL of Tris buffer, centrifuged at 13,000 rpm for 5 min to collect the supernatant, and stored at 4 °C until use. The amount of immobilized lipase on HNTs was calculated by obtaining the difference in the amount of protein contents in the supernatant before and after adsorption. The catalytic activity of immobilized lipase was also determined and compared with free lipase by measuring the hydrolysis of p-nitrophenol palmitate by using previously reported spectrophotometrical method [34]. Moreover, different concentrations of lipase (1 mL, 0.5, 1, 1.5, 2, 3, and 4 mg mL^{-1}) were incubated with a constant amount of HNTs (40 mg) to optimize the enzyme concentrations during the adsorption process. In addition, different pH values (5.6, 6.2, 6.8, 7.4, and 8.0) and temperatures (4, 25, and 37 °C) of the incubation solution were used to investigate the extent of enzyme adsorption. The stability of lipase-immobilized nanotube reactor was evaluated by comparing its catalytic capability with free lipase after 2 weeks' storage.

2.4. Optimization of operative parameters for lipase immobilized-HNTs based ligand screening

To test the specificity and to select the best conditions for ligand screening of the lipase-immobilized HNTs, a self-made test mixture containing orlistat (a lipase inhibitor with IC_{50} of 0.423 μM) and schisandrin (a phytochemical without lipase inhibitory activity) was used. Briefly, lipase-immobilized HNTs were incubated with the mixture (1 mL, containing 2 mg mL^{-1} orlistat and 0.2 mg mL^{-1} schisandrin) for 2 h, followed by three times of washing with 1 mL of Tris buffer (ionic strength varied from 150 mM to 1000 mM; pH varied from 5.6 to 8.0). Degeneration solvent (10–100% acetonitrile) was added to release the potential ligands from the enzyme-loaded HNTs, whereas empty HNTs were operated in the same manner to exclude non-specific adsorbed compounds. The specificity of ligands screening was determined by the binding degree which was calculated by the following formula: binding degree = $(A - A_0)/A_{total}$, where A and A_0 are the peak areas of the compound in the degeneration solution of lipase-coated HNTs and the empty HNTs, respectively. A_{total} represents the peak area of the compound in the original test mixture. The detailed experimental conditions on the analysis of the mixture are found in Supporting Information S1.

2.5. Screening potential ligands from Magnoliae cortex using lipase immobilized HNTs

The screening of potential lipase ligands from the extract of *Magnoliae cortex* was performed as follows. A 1 mL extract solution (5 mg mL^{-1}) was incubated with lipase-immobilized HNTs (40 mg, dissolved in 1 mL pH 6.8 Tris buffer) for 2 h at room temperature. After washing three times with 1 mL of Tris buffer (to remove unbound compounds), the potential ligands were then released by 1 mL of 50% acetonitrile and followed by centrifuging for 5 min. The supernatants were injected into the HPLC–MS. Two groups were designed for this experiment, namely, the sample group using the lipase-immobilized HNTs and the control group using the blank HNTs, to distinguish the non-specific binding compounds.

The HPLC–MS analysis of degenerated samples was performed on an Agilent 1100 series HPLC system connected to the LCQ DecaXP^{plus} mass spectrometer via an ESI source. Agilent Zorbax SB-C_{18} column (4.6 mm, 250 mm, 5 μm) was used for separation. The flow rate was 0.8 mL min^{-1}, and the column temperature was set at 30 °C. The mobile phase was 0.05% formic acid–water (A) and acetonitrile (B). The linear gradient program was as follows: 0/20,