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Effect of pore diameter and cross-linking method on the immobilization efficiency of *Candida rugosa* lipase in SBA-15

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ABSTRACT

The influences of pore diameter and cross-linking method on the immobilization efficiency of *Candida rugosa* lipase (CRL) in a mesoporous material have been investigated. Five kinds of SBA-15 with different pore-sizes (6.8 nm, 9.1 nm, 13.2 nm, 15.6 nm and 22.4 nm) were chosen as the carrier. The one with pore diameter of 15.6 nm was proved to be a more suitable immobilization support than the others, and the loading amount reached 343.6 mg/g. To solve the leaching problem of the adsorbed enzyme, chitosan and glutaraldehyde were used as the "bridge unit" and "cross-linking agent", respectively, which were supposed to realize the immobilization of lipase molecules adsorbed on the surface of pores into a mesh-like layer. The experimental results showed that the activities of the immobilized CRL were much higher than that of free lipase and remained 80.5% of the initial activity after 6 cycles in 48 h.

1. Introduction

Recently, mesoporous silica materials have gathered a significant attention in both academic and industrial areas because of its well-ordered and adjustable pore structures as well as abundant surface silanol groups. Their large surface area and high pore volume may facilitate the entry of enzyme molecules, enable the reusability of enzymes and enhance their activity, selectivity as well as operational stability, thus they have been especially used in the area of enzyme immobilization. Up to now, the immobilization of lipase on several kinds of mesoporous silica, such as MCM-41, MCM-48, MCF, SBA-15, has been well investigated (Lee et al., 2005; Hartmann, 2005; Kim et al., 2007; Li et al., 2009a).

It has been reported that the pore-size of support materials, especially the ratio of pore-size to the enzyme molecule size, may greatly influence the enzyme adsorption (Lu et al., 2008; Kang et al., 2007). The ideal size of pores was found to be 3–5 times of the protein size. (Serra et al., 2008; Gritti and Guiochon, 2007). Pandya et al., 2005) also pointed out that pore diameters of the mesoporous materials, such as MCM-41, SBA-15, MCF-153, had a great impact on the activity of immobilized α-amylase, mainly because only the external surface of the material with small pore could absorb the enzymes, resulting in a low enzyme loading. On the other hand, the results reported by Kang et al. (2007) showed that too large pore may lead to the leaching out of lipases from the channels in the adsorption process, which also accounted for a decrease in enzyme loading. They also found that the most suitable pore diameter for *Porcine pancreatic* lipase (PPL) immobilization was 13 nm. As proved in many researches, pore-size is probably the most important parameter in the enzyme immobilization process, while other textural properties, such as the nature of the pores (channel-like or cage-like), the connectivity of the porous network, total pore volume as well as surface area, do not have obvious effects (Serra et al., 2008). However, since the enzymes used as model proteins were still limited, the theoretical relationship between the enzyme size and the suitable pore-size has not been systematic studied. Therefore, the determination of suitable pore-size for different lipase still relies on more experiments. *Candida rugosa* lipase (CRL), with a molecular weight of 45,000–60,000 and a molecular volume of $5 \times 4.2 \times 3.3$ nm^3, was one of the most widely used lipases that catalyze the hydrolysis of triglycerides. However, as far as we know the suitable pore-size for CRL immobilization has not been determined yet. In this work, five different pore diameters of SBA-15 (6.8 nm, 9.1 nm, 13.2 nm, 15.6 nm and 22.4 nm) prepared with the method proposed by Zhao et al. (1998) were used as the CRL supporters, providing a possibility for a systematic study of the influences of pore-size on lipase immobilization process.

Another problem worth noticing is the stability of immobilized enzymes. While the adsorption method is simple to perform, the stability is poor due to the leaching of adsorbed enzymes, which may cause an uneasy judgment of whether the measured activity belongs to the immobilized lipase or the free one, especially when using a hydrophilic material as the support for aqueous phase catalysis. For example, Serra et al. (2008) studied the loss of the adsorbed lipase and found that nearly 30% of the enzyme was leached

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from the support within 2 h. Yiu et al. (2001) also reported that 35–72% of the adsorbed trypsin on MCM-41, MCM-48 and SBA-15 leached from the support after stirring in the buffer solution for 2 h. Therefore, it is necessary to improve the immobilization stability. The conventional approach employed most frequently for the enzyme immobilization into the mesoporous silica was covalent attachment (Wu et al., 2009; Huang et al., 2008). However, the only existed groups on the surface of silica are hydroxyl groups which are difficult to form chemical bonds with enzyme molecules directly. Some linking agents such as glutaraldehyde cannot play role in the immobilization procedure either, because of a lack of amino group on the support. Therefore, the silica support was always modified with various functional groups (generally $-NH_2$ or –CHO group) as to generate chemical bonds with lipase; however, it required multi-step synthetic processes or harsh conditions (Hong et al., 2008; Sulek et al., 2010). For example, Bai et al. (2006) realized the modification process of mesoporous silica by four steps, using methane sulfonic acid and ultrasonic vibration for pre-treatment, refluxing the unmodified silica for 24 h in anhydrous toluene under vigorous stirring, and the reaction temperature was 80 °C for 10 h. Moreover, since not all the hydroxyl groups existing on silicas can be replaced by the functional group, there would be a waste of surface area and pore volume in case the modification is not complete. It can be speculated that if the lipase molecules happened to adsorb into the support with no functional group, no chemical bond would form, still leading to the loss of enzyme in aqueous phase. This kind of cross-linking method may be considered as "point cross-linking" (see Fig. 1(a)) and cannot take full advantage of the mesoporous materials. Therefore, the new methods combining the advantages of adsorption and covalent attachment are in urgent need.

To solve the leaching problems of the adsorbed lipase and improve the conventional way for lipase immobilization, a novel and easy cross-linking method was proposed. Firstly, SBA-15 was used for lipase adsorption, and these adsorbed enzymes were not stable enough because there was only weak H-bond between Si–OH and NH_2 group in the lipase molecules. Then chitosan (CS), an abundant long-chain polymer with enough NH_2 groups was added and was supposed to adsorb on and into the pore of mesoporous silica. After the addition of the cross-linking agent (glutaraldehyde), the Schiff reaction took place and the chitosan molecules would connect with each other through NH_2 group and glutaraldehyde, more importantly, the lipase molecules would also be cross-linked in the chitosan "mesh" through their own NH_2 groups (Fig. 1(c)). Therefore, a large and stable network covering both the external and internal surface of the support among the lipase and chitosan molecules was formed (see Fig. 1(b)). The method was very different from the conventional method in which the support was modified firstly, and then a covalent bond formed between the support and the lipase molecules. In this method, no chemical bond was generated between lipase and the mesoporous silica, and chitosan was speculated to play the role of "bridges" and attach the adsorbed lipase. This method was supposed to be "surface cross-linking" since all the surface of SBA-15 had equal opportunity for lipase adsorption and immobilization as well. It also showed a good stability in the following experiment, which indicated the feasibility of the new immobilization method.

2. Methods

2.1. Materials

Candida rugosa lipase type VII was purchased from Sigma–Aldrich (Dorset, UK); glyceryl triacetate was purchased from Guang Fu Fine Chemistry Institute (Tianjin, China); Chitosan (CS, MW = 4.9×10^5, deacetylating degree 94.5%) was purchased from YuHuan Chemical Company (Zhejiang Province, China). Tetraethyl orthosilicate (TEOS) and Ammonium fluoride were obtained from Xilong Chemical (Shantou, China). P123 triblock copolymer (poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide), EO20-PO70-EO20, Mav = 5800) was obtained from Will Chemical Co., Ltd. (Nanjing, China). Hydrochloric acid (HCl, 36.5 wt.%) was produced by Beijing Chemical Company (Beijing, China). All the reagents were directly used without any further purification. All the solutions were prepared with the distilled deionised water.

2.2. Methods

2.2.1. Preparation of SBA-15

Firstly, 2 g of triblock copolymer P123 was dissolved in 75 ml of the hydrochloric acid solution with the concentration of 1.6 M, and then appropriate amount of pore-expanding agent was added to adjust the final target pore-size, and the solution was heated to 39 °C and magnetically stirred for 3 h. After that, 4.5 ml of tetraethoxysilane was dropped into the solution and stirred for another 24 h, then 23 mg of ammonium fluoride in water (2.5 ml) was added to the solution, it was transferred to an autoclave for aging at 100 °C for 30 h. The obtained precipitate was filtered, washed with water and ethanol, dried and calcined at 550 °C in a Muffle furnace for 6 h. The synthesized SBA-15 consisted of the small rod-like particles of 1–2 μm in length which were further aggregated into a wheat-like structure.

2.2.2. Characterization

Nitrogen adsorption–desorption isotherms were measured at 77 K using a Quantachrome Autosorb-1-C Chemisorption–Physisorption Analyzer after the SAB-15 samples (0.015 g) were outgassed for 2 h at 200 °C. SBA-15 with adsorbed or cross-linked lipase (0.015 g) was outgassed for 6 h at 80 °C. The BET surface area was calculated from the adsorption branches in the relative pressure range of 0.10–0.30, and the total pore volume was evaluated at a relative pressure of about 0.995. Thermogravimetry measurement was carried out on a TG 2050 analyzer, and samples were prepared by adding 0.05 g of SBA-15 into 25 ml of phosphate buffer or lipase solution for 24 h and then cross-linking with chitosan (detailed procedure was shown in Section 2.2.3). The TG measurement of synthesized SBA-15 was also performed for comparison. Scanning electron microscopy (SEM) observations were performed on a Hitachi S-450 microscope operating at 20 kV and a JEOL JSM 7401F microscope operating at 1.0 kV.

2.2.3. Immobilization of lipases in SBA-15

Lipase solutions were prepared by adding appropriate amounts of lipase powder to phosphate buffer (pH = 7.0, 0.05 M). The adsorption experiments were carried out by mixing 50 mg of SBA-15 with 25 ml of lipase solution, and the mixture was magnetically stirred at room temperature for 24 h. After that, 0.5 ml of the mixture was taken out and centrifuged for enzyme concentration measurement, then 1 ml of CS solution, prepared by dissolving 0.5 g of CS powder in 100 ml of 0.5% v/v hydrochloric acid (HCl, 38%), together with 1 ml of 1.0 wt.% glutaraldehyde was added into the solution, the new mixture was stirred for another 20 min at room temperature to cross-link the adsorbed lipases with CS molecules. Finally, the mixture was centrifuged to separate the support with cross-linked lipase from the solution, and 0.5 ml of the supernatant, whose total volume was calculated as 27 ml, was taken out for protein concentration measurement to determine the amount of immobilized lipase. The support with lipase was washed 3 times to get rid of impurities before activity assay.