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Synergistic effects of amine and protein modified epoxy-support on immobilized lipase activity

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ABSTRACT

We have developed an improved and effective method to immobilize *Yarrowia lipolytica* lipase Lip2 (YLIP2) on an epoxy poly-(glycidylmethacrylate-triallyisocyanurate-ethyleneglycoldimethacrylate) ($P_{GMA\text{-}TAIC\text{-}EGDMA}$) support structure with or without amine or/and protein modifications. Our results show that there is an increase in the activity of the immobilized lipase on n-butylamine (BA) modified support (420 U/g support) and the biocompatible gelatin modified support (600 U/g support) when compared to the support without modification (240 U/g support). To further study the influences of BA and gelatin modification on the activity of the immobilized lipase, gelatin and BA were concurrently used to decorate the support structure. Lipase immobilized on 2% BA/gelatin (1:1) modified support obtained the highest activity (1180 U/g support), which was five-fold higher than that on a native support structure. These results suggest that the activity of a support-immobilized lipase depends on the support surface properties and a moderate support surface micro-environment was crucial for elevated activity. Collectively, these data show that a combined gelatin and BA modification regulates the support surface more suitable for immobilizing YLIP2.

1. Introduction

Lipases are a group of enzymes that have great applications in food, pharmaceutical, cosmetic, and chemical industries [1–4]. However, lipase-based catalysts are usually expensive, unstable, and are difficult to be efficiently separated from reaction systems used in industrial production [5]. To address this, various immobilization techniques have been developed to improve their stabilization, reutilization [6,7] and other properties [8–10]. For example, the enzyme aggregation reduced in the immobilization processes compared to free enzyme and generated favorable environment [11,12]. Multipoint covalent attachment of enzyme on highly activated pre-existing supports via short spacer arms involving many residues placed on the enzyme surface promotes rigidification of structure of the immobilized enzyme [13]. For some enzymes with several subunits, the immobilization process may protect the stabilization and prevent subunit dissociation [14].

However, a simple protocol for effective immobilization system is not easily accomplished because small changes in variables such as nature and characteristics of the immobilization support as well as age in vs coupling chemistry can have a significant impact on biocatalyst activity and stability [15,16]. Therefore, it is very important to select a suitable support and design proper modification protocols, especially to create the appropriate micro-environment for enzymes in the supports, and it is still challenging to develop optimized immobilization techniques.

Currently, there are four main immobilization techniques: covalent binding, adsorption, cross-linking, and entrapment [5]. Importantly, immobilized lipases can become more active than their native form. This is due to the fact that the lipase can exist in two forms: an open, *active* form in which the lid displaces and allows substrate access to the catalytic site and a closed, *inactive* form in which the lid shuts, thereby hiding the catalytic site [17–19]. In aqueous media, the lid exists in a closed conformation. However, in the presence of an insoluble substrate, the lipase becomes active by interfacial activation, shifting lid conformation from a closed to an open state [17]. Lipase becomes more active if the interactions between them and their support stabilize their open form which generates a higher activity [20].

Immobilization techniques utilizing hydrophobic matrices have attracted much attention, since the interaction between the lipase and its hydrophobic support is similar to the phenomenon observed with insoluble substrates [21]. Moreover, the accessibility of

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the substrate to the active site of the lipase lid was promoted by employing hydrophobic support [22]. Lipase immobilized on hydrophobic supports involves its open form of the lipase, and the hydrophobic support makes lipase more stable [23]. Thus far, there are two strategies to make hydrophobic supports. One strategy is that the support itself may be reagent (e.g. acccurel, divinylbencene, etc.) [24,25]. The other is to decorate the support with a hydrophobic reagent [26]. For instance, Jin et al. [27] modified magnetic siliceous mesocellular foam (MCF) with organosilane to improve the support surface's hydrophobicity. They found that lipase derived from *Pseudomonas cepacia* (PCL) that was immobilized on a support of hydrophobic MCF-Ph obtained the highest activity, which was eight times that of native MCF. Taken together, results obtained from time-resolved fluorescence catalytic activity experiments showed that the hydrophobic activation drove the improvement in catalytic activity [16].

However, the excessive support resulting from this hydrophobic interaction can destroy the structure of the enzyme, leading to rapid denaturation and a sharp decrease in the activity of surface-bond enzymes [28]. Ultimately, this excess would decrease the enzyme properties [25]. When compared with hydrophobic supports, lipase immobilized on hydrophilic supports can acquire a higher efficiency of immobilization. When a lipase which was immobilized on a hydrophilic support was used in organic solution, water was able to concentrate around the lipase, thus promoting the catalysis of the reaction [29,30]. In addition, the process of tethering enzymes to carriers could cause inevitable conformational changes of the enzymes, resulting in decreased bioactivities of enzymes [31].

To overcome this limitation, researchers have adopted many methods, one of which is to modify the support surface with bio-friendly molecules to obtain the biocompatible surface to reduce the enzyme denaturation during the immobilization [32–34]. There are many methods to form such biocompatible ("bio-friendly") support surfaces, including coating, adsorption, and self-assembly [35,36]. Among these methods, modifications to support structures, such as natural, molecular-like gelatin, collagen, alginate, or biotin on the support have often been used in tissue engineering due to their non-toxic nature [37].

In this work, the support $P_{GMA\text{-}TAIC\text{-}EGDMA}$ with epoxy group was used as the immobilized support while the epoxy group could be modified by thiols, amino, hydroxyl and carboxyl groups easily, and the final bonds are very stable. However, the epoxy group exhibits a relative low reactivity [13]. Thus, many support modifications have been researched [38–40]. Although there are many hydrophobic and biocompatible modifications for supports, most have only used hydrophobic or bio-friendly reagents to regulate the support environment. In our study, different types of amines and proteins were introduced to investigate their respective activities in our first work. Lipase could be immobilized on support by different modifications such as ionic exchange, hydrophobic adsorption, covalent binding, and biocompatible adsorption. Here we present work in which we prepared an amine and protein immobilized matrix in order to regulate the properties of the support surface. The ratios of gelatin to BA, the concentrations of BA/gelatin and the sequences of BA and gelatin modified support were studied, and the synergistic effects of the hydrophobic, ionic and biocompatible modifications on the activity of the immobilized lipase were analyzed.

2. Materials and methods

2.1. Materials

Both the *Yarrowia lipolytica* lipase YLIP2 lipase and the epoxy support $P_{GMA\text{-}TAIC\text{-}EGDMA}$ were previously produced in our laboratory [41,42]. Gelatin, fibrion, casein, ovalbumin, bovine serum albumin, BA, methylamine, and n-octylamine were purchased from Beijing Chemical Factory (Beijing, China). All of the experimental reagents and solvents used were of analytical grade.

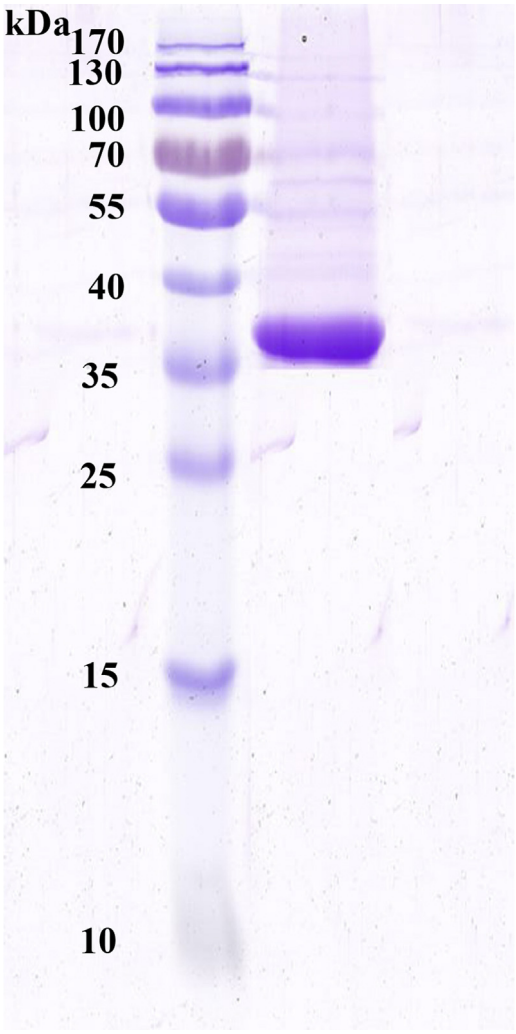

Fig. 1. SDS-PAGE of *Yarrowia lipolytica* lipase lip2.

2.2. The preparation of lipase solution

The fermentation of YLIP2 lipase in our laboratory was carried out in a 5 L reactor at 26 °C with stirring at 500 rpm and with an air input of 1 VVM (air volume/culture volume/min). The culture medium was composed of 4% (wt.) soybean powder, 4% (wt.) soybean oil, 0.1% (wt.) KH_2PO_4 and 0.1% (wt.) $(NH_4)_2SO_4$. The lipase produced reached 8000 U/ml after 96 h fermentation. The cells were removed by centrifugation, and the lipase in the supernatant was precipitated by addition of three volumes of acetone. The precipitate was washed with acetone and dried at room temperature. The activity of the lipase powder is 67000 U/g. The lipase powder was dissolved into distilled water getting the lipase solution (10 mg/ml). The purity of YLIP2 lipase is higher than 90%, which was confirmed by SDS-PAGE via Quantity-One (Fig. 1).

2.3. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on protein using a Mini Protein Electrophoresis Cell (Bio-Rad Laboratories, California, USA) and a 12% acrylamide separating and a 5% acrylamide stacking. Protein