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Immobilization of lipase from *Candida rugosa* on electrospun polysulfone nanofibrous membranes by adsorption

Zhen-Gang Wang [a,b], Jian-Qin Wang [a,b], Zhi-Kang Xu [a,b,*]

[a] *Institute of Polymer Science, Zhejiang University, Ministry of Education, Hangzhou 310027, PR China*
[b] *Key Laboratory of Macromolecule Synthesis and Functionalization (Zhejiang University), Ministry of Education, Hangzhou 310027, PR China*

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Abstract

Polysulfone composite nanofibrous membranes were prepared by electrospinning and were used to immobilize lipase from *Candida rugosa* by physical adsorption. Field emission scanning electron microscopy was used to evaluate the morphology and diameter of the nanofibers. PVP and PEG were used as additives to render the nanofibrous membranes biocompatibility favored by immobilized enzyme. Effects of post-treatment, additive concentration, pH and temperature were investigated on the adsorption capacity and activity of immobilization preparations, as well as thermal stability. It was found that (1) post-treatment had no significant effect on the adsorption capacity; (2) the increment of PVP or PEG concentration was negative for the adsorption capacity but positive for activity of immobilized lipase; (3) the immobilized lipase showed less sensitivity for pH and higher optimum temperature. Thermal stability for the immobilization preparations was enhanced compared with that for free preparations. The kinetic parameters of the free and immobilized lipases, K_m and V_{max} were also assayed. Results indicated that K_m and V_{max} for the immobilized lipases were higher and lower than those for free lipase, respectively.

Keywords: Electrospinning; Polysulfone; Nanofibrous membrane; Enzyme immobilization; Lipase; Adsorption

1. Introduction

Enzymatic biotransformations have been pursued extensively for a wide range of important chemical processing applications, largely as a result of their unparalleled selectivity and mild reaction conditions [1]. In many cases, however, the low catalytic efficiency and stability of enzymes are considered as barriers for the development of large-scale operations and applications. The performance of immobilized enzyme depends greatly on the characters and structure of the carrier materials. Many efforts have been concentrated on modifying the carriers, in order to make the carriers more suitable for enzyme immobilization and catalysis, such as rendering biocompatibility, hydrophilicity, etc. [2–6]. Nevertheless, even for the modified supports, the enzyme loading is usually considerably low. Alternatively, high enzyme loading can be achieved with porous materials such as membranes, gel matrices, and porous particles [1,7–13]. Recent efforts using nanostructured materials are an intriguing approach since all these materials can provide a large surface area for the attachment of enzymes [14]. Among them, electrospun nanofibers show some attractive prospects, compared to the other nanostructures. On one hand, nanofibers can relieve remarkably diffusion resistance of the substrates/products, due to the shortened path of diffusion resulting from the reduction of the geometric size of the enzyme support, compared to porous materials; on the other hand, the nanofibers, which can be processed into various structures such as non-woven mats, or well-aligned arrays, are more conveniently recovered and more durable than other nanoparticles or carbon nanotubes. Furthermore, electrospinning is simple and versatile. Based on the above advantages, electrospun nanofibers have attracted a great deal of attention as enzyme carriers [1,14–19].

Lipase (triacylglycerol acyl ester hydrolyses, EC 3.1.1.3) is an enzyme possessing an intrinsic capacity to catalyse the cleavage of carboxy ester bonds in tri-, di-, and monoacylglycerols to glycerol and fatty acids [20]. The enzyme is distributed among higher animals, plants and micro-organisms in which it plays a key role in the lipid metabolism. Moreover, a promising property

* Corresponding author. Tel.: +86 571 8795 2605; fax: +86 571 8795 1773.
E-mail address: xuzk@zju.edu.cn (Z.-K. Xu).

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of lipases is their activation in the presence of hydrophobic interface [21], and in this case, important conformational rearrangements take place, yielding the "open state" of lipases. Among kinds of materials, polysulfone showed some interests, due to its hydrophobicity, facile process, and chemical inertness. However, the poor biocompatibility of this material may cause nonbiospecific interaction, protein denaturation, and enzyme activity loss. Several approaches have been provoked to modify the materials, such as surface modification by grafting [22], or blending with other materials, such as PVP [23], to introduce a biofriendly interface, which may benefit the enzyme activity. In addition, polysulfone has been successfully electrospun into nanofibers [24,25], and, to our knowledge, polysulfone nanofibers were rarely used to immobilize enzymes. Hence, PSF/a biocompatible polymer electrospun composite nanofibers were adopted to immobilize lipase, in order to investigate the effect of the composite nanofibers on the behavior of the lipase.

In this study, model enzyme, lipase, was immobilized onto electrospun polysulfone nanofibers by physical adsorption, considering the hydrophobic character of PSF. The immobilization behavior and kinetic parameters were discussed with various additives in the spinning solution.

2. Experimental

2.1. Materials

Polysulfone (PSF) (from Shuguang Engineering Materials factory, Shanghai, China) possessing viscosity average molecular weight of 4.7×10^4 was used after dried in a vacuum oven. Poly(*N*-vinyl-2-pyrrolidone) (PVP) and poly(ethylene glycol) (PEG200) were used as received; their weight average molecular weights were 58,000 and 190–210 g/mol, respectively. *Candida rugosa* lipase (type VII, 26.2 U/mg protein) and *p*-nitrophenyl palmitate (*p*-NPP) were of biological grade and purchased from Sigma. Coumassie brilliant blue (G250) for the Bradford protein assay was from Urchem and BSA (Bovine Serum Albumin, BP0081) from Sino-American Biotechnology. All other chemicals were of analytical grade and used without further purification.

2.2. Preparation of electrospun polysulfone composite nanofibrous membranes

Weighted PSF powder was dried in a vacuum oven at 80 °C for about 24 h, and then dissolved in *N*,*N*-dimethylacetamide (DMAC) at 120 °C with vigorous stirring to form homogeneous solution with PVP (or PEG200). PSF concentration was always 18 wt.% in the solution. Electrospinning was carried out using a syringe with a 1.2 mm diameter spinneret at an applied electrical potential difference of 10 kV over the 10 cm gap between the spinneret and the collector. The syringe pump was set to deliver the solution at a flow rate of 1.0 mL/h using a 20 mL syringe. It usually took 3 h to obtain a thick membrane that could be detached from the tinfoil collector. The electrospun membranes were dried in the vacuum oven at 80 °C for at least 5 h to remove the residual solvent before used.

2.3. Morphology observation

Field emission scanning electron microscopy (FEI, SIRION-100, USA) was applied to evaluated the morphology and diameter of PSF composite nanofibers at 5 kV. Before analysis, the samples were sputtered with gold using Ion sputter JFC-1100.

2.4. Immobilization of lipase onto the nanofibrous membrane by adsorption

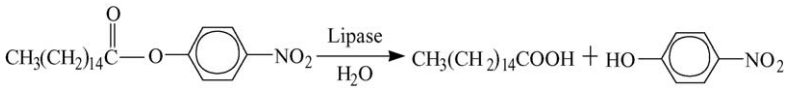
An appropriate amount of electrospun membranes was immersed in ethanol for about 3 h (to increase the wettability of the membrane), thoroughly washed with deionized water to remove the residue ethanol, and then rinsed with phosphate buffer solution (50 mM, pH 7.0). Subsequently, the pretreated membranes were submerged into the lipase solution (10 mg/mL in the buffer, 50 mM, pH 7.0) in a 25 mL beaker and shaken gently in a water bath at 30 °C for the required time. Finally, the membranes were taken out and washed with the buffer until no protein was detected in the washings.

2.5. Determination of immobilization capacity

Protein concentration in the solutions was determined with Coumassie Brilliant Blue reagent by the method of Bradford [26] using BSA as protein standard, on UV–vis spectrophotometer (756PC, Shanghai Spectrum Instruments Co. Ltd.). The amount of immobilized enzyme protein was estimated by subtracting the amount of protein determined in the filtrate and washings from the total amount of protein used in immobilization procedure. Lipase adsorption capacity of the membrane was defined as the amount of protein (mg) per gram of the fibrous membrane.

2.6. Activity assays of free and immobilized enzyme

The reaction rate of the free and immobilized lipase preparations was determined according to the method reported by Chiou [27], also described in our previous work [6]. In the standard conditions, the reaction mixture was composed of 1.0 mL ethanol containing 14.4 mM *p*-NPP and 1.0 mL phosphate buffer solution (PBS) (50 mM, pH 7.5) in an Erlenmeyer flask. The reaction was started by the addition of 0.1 mL free lipase preparation (or 25 mg immobilized lipase preparation), and the scheme is indicated as follows.



The mixture was incubated at 37 °C under reciprocal agitation at a certain stroke rate. After 5 min, the reaction was terminated

by adding 2.0 mL of 0.5N Na_2CO_3 followed by centrifuging for 10 min (10,000 rpm). The supernatant of 0.50 mL was diluted 10-fold with deionized water, and measured at 410 nm in an UV–vis spectrophotometer. The reaction rate was calculated from the slope of the absorband versus the time curve. Molar extinction coefficient was adopted as $14.5 \times 10\,M^{-1}\,cm^{-1}$ for *p*-nitrophenol (*p*-NP), which was determined from the absorbance of standard solutions of *p*-NP in the reaction medium.

One enzyme unit was the amount of biocatalyst liberating 1.0 μmol of *p*-NP per minute in these conditions. The enzyme activity was the number of lipase unit per gram membrane. Specific activity was defined as the number of lipase unit per milligram protein. Activity retention was defined as the ratio of the activity of the amount of the enzyme coupled on the electrospun membrane to the activity of the same amount of free enzyme.

2.7. Thermal stability

Free and immobilized lipase preparations were stored in phosphate buffer solutions (50 mM, pH 7.0) at 50 °C for 100 and 120 min, respectively. 0.1 mL free lipase solutions (1.0 mg/mL) or a certain amount of immobilized membranes were periodically withdrawn for activity assay. The residual activity was determined as above.

3. Results and discussion

3.1. Effect of adsorption time on adsorption capacity

To determine the moderate adsorption time, the effect of adsorption time on the immobilized amount of protein was studied, as shown in Fig. 1. The immobilized amount increased as adsorption time increased until it reached about 90 min, which might be at the point of dynamic balance between adsorption and desorption. It took relatively much time to reach the balance than reported [28,29], which mainly depended on the adsorption condition. The contact between the nanofibrous membrane and lipase in the solution during gently shaking, and the relative low temperature which lowered the diffusion of lipase from the solution to nanofiber surface both required delayed balance time. Therefore, to ensure the full adsorption of lipase onto the membranes, the adsorption time was adopted as 90 min in the following experiments (considering the small difference among the membranes electrospun under various conditions, the following measurements were also carried out using adsorption time of 90 min).

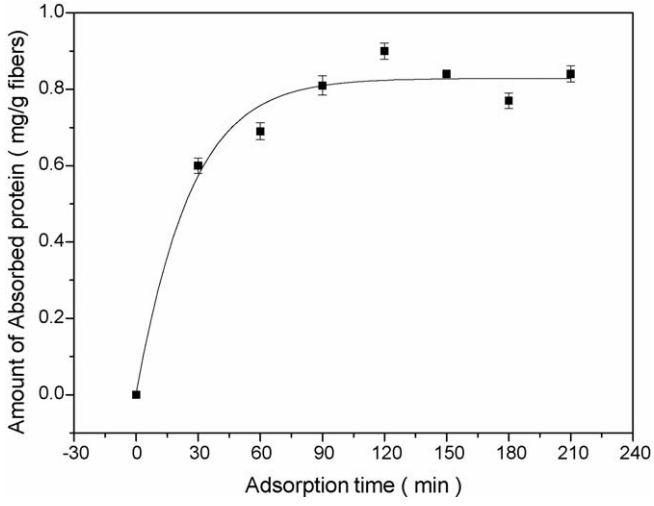


Fig. 1. Effect of adsorption time on enzyme loading capacity. Carriers: pure PSF nanofibers with diameters of 257 ± 45 nm. Process temperature: 30 °C.

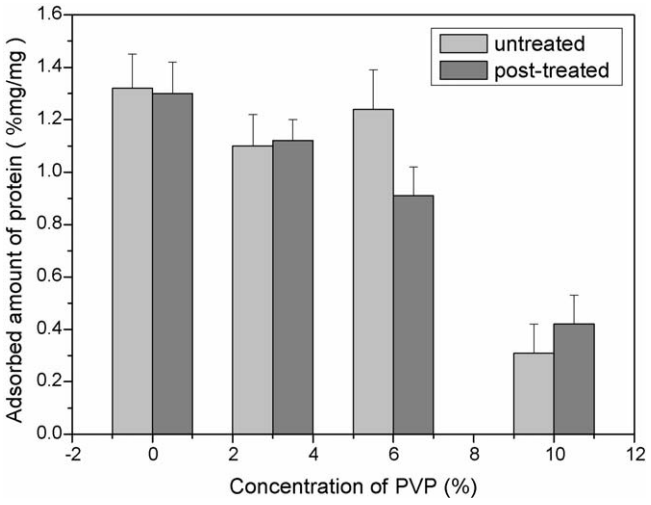


Fig. 2. Effect of post-treatment on the amount of immobilized lipase absorbed on the PSF nanofibrous membrane containing different amount of PVP.

3.2. Effect of post-treatment on adsorption capacity

The specific surface area could affect the amount of bound enzyme a lot, especially for the immobilization by physical adsorption. Usually, the cradle for enzyme immobilization with more porosity was believed to possess larger surface area. Therefore, using PSF nanofibers as the fundamental, post-treatment was used to endow the fiber surface with porous structure. Herein, the treatment was carried out by doping PSF/PVP composite nanofibers in the hypochlorite solution at 30 °C, and shaking for 24 h. The amount of bound lipase on the PSF nanofibrous membrane before and after hypochlorite treatment was indicated in Fig. 2. No significant change was observed in lipase immobilization, although the surface of treated fibers showed more porous compared with that untreated one (Fig. 3), which could be attributed to the reaction between hypochlorite and PVP that caused the chain scission of PVP molecules and the eventual leaching of PVP from the fiber matrix [30]. It might be because that the pore size on the fiber was not big enough to accommodate the approached lipase only by diffusion.

3.3. Effect of additive content on the adsorption capacity and activity of lipase immobilized on the nanofibers

As PVP or PEG200 content increased, the amount of protein mainly showed a decreasing tendency. PEG200 or PVP

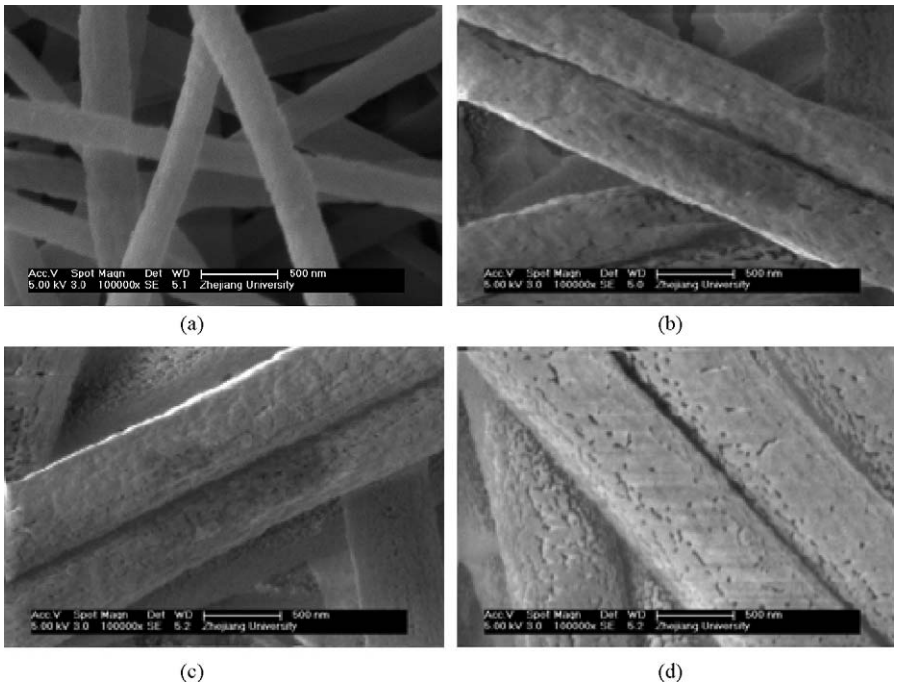


Fig. 3. Morphologies of post-treated PSF/PVP nanofiber. PSF concentration in the spinning solution: 18 wt.%. PVP concentration: (a) 0 wt.%; (b) 3 wt.%; (c) 6 wt.%; (d) 10 wt.%. Treating time: 24 h. Treating temperature: 30 °C.

addition could increase the viscosity of PSF solution, which would increase the fiber diameter, followed by decreased the effective area for lipase adsorption. However, PEG was usually used as electrical conductivity enhancer [31–33], which would be negative for the increment of the PSF/PEG composite nanofibers [34]. The competition between electrical conductivity and solution viscosity might result in the maximum fiber diameter as PEG concentration increased. In addition, the addition of PVP or PEG200 could endow the fiber surface hydrophilicity. Lipases possess a promising property of activation by hydrophobic interfaces, which has been recognized as a common feature up to now. In the absence of interfaces, lipases have some elements of secondary structure (termed the 'lid') covering their active sites and making them inaccessible to substrates. However, in the presence of hydrophobic interfaces important conformational rearrangements take place yielding the 'open state' of lipases. These rearrangements result in the exposure of hydrophobic surfaces, the interaction with the hydrophobic interface, and the corresponding functionality on the enzyme. According to the complex multiple conformation properties of lipases [35,36], the interfacial hydrophobic interaction between the support surface and the hydrophobic domain around lipase's active center seems to dominate the adsorption strength. Therefore, the more hydrophilic surface might also weaken the lipase adsorption capacity on the nanofibers.

Increasing PVP or PEG content resulted in increasing the activity of immobilized lipase. PVP and PEG were widely used in the medicine and biotechnology because of their excellent biocompatibility and they were often utilized as additive in membrane industry due to their good water-solubility [23,37]. Herein, it was possible that the biocompatible additive could reach the fiber surface during the process of lipase immobilization operation, which would provide a compatible microenvironment for the protein to retain its natural conformation, which in turn benefited its activity. Giving both attention to enzyme loading capacity and enzyme activity, in the following discussion, the used supports were the membranes electrospun from the spinning solution containing 6 wt.% PVP and the fibers diameter was determined as 542 ± 77 nm.

3.4. Effect of pH and temperature on the activity of adsorbed lipase

Fig. 4 shows the effect of pH on the activity of the free and the immobilized lipases. The optimum pH value for the free lipase was about 7.7, while that for the immobilized lipase on the PSF/PVP nanofibrous membrane shifted to neutral region at about 7.0. These observations suggested a significant alteration of enzyme microenvironment upon immobilization on the fibers and consisted with documented literature [38]. The alteration might be attributed to enrichment of PVP on the fiber surface. It is well known that PVP is one kind of polycation, which resulted in the more alkaline microenvironment for absorbed lipase than that for free lipase. It made the optimum pH in the solution more acidic. As also shown from Fig. 7, immobilized lipase was less sensitive to pH changes at alkaline pHs than acidic pHs compared to that of free lipase.

The effect of temperature on the activity of free and immobilized lipases for *p*-NPP hydrolysis at pH 7.0 in the temperature range of 25–55 °C is shown in Fig. 5. It was found that the immobilization shifted the optimum temperature for lipase activity

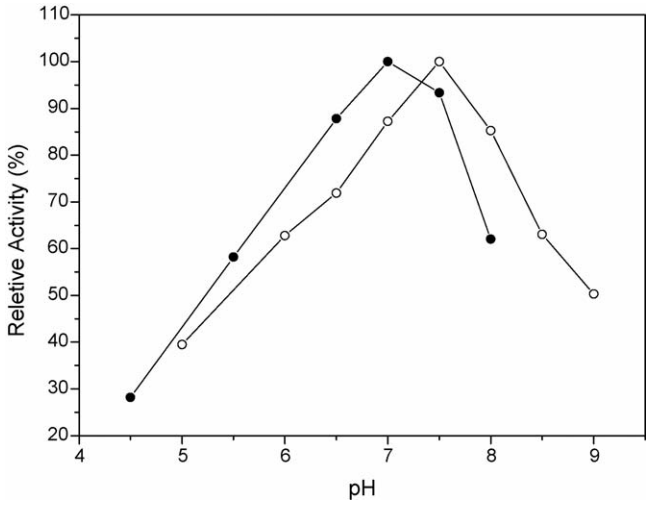


Fig. 4. Effect of pH on the activity of lipase: (◯) free lipase; (●) adsorbed lipase on PSF/6 wt.% PVP nanofibers. Reaction temperature: 37 °C.

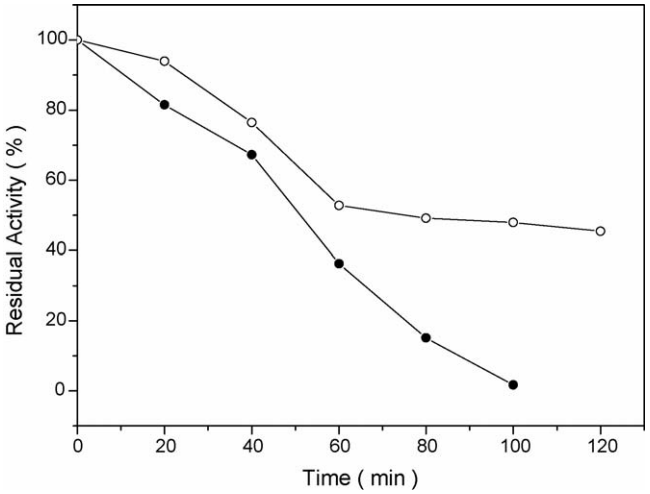


Fig. 6. Thermal stability of free and immobilized lipase: (●) free lipase; (◯) adsorbed lipase on PSF/6 wt.% PVP nanofibers.

from about 35 °C of free enzyme to 45 °C for the immobilized enzyme. This was either due to creation of conformational limitation on the enzyme movement as a result of adsorption strength between the supports and the enzyme or a low restriction in the diffusion of the substrate at high temperature. In addition, the improved resistance of protein to thermal denaturation is also an important factor. After all, the immobilized enzymes showed their catalytic activities at a higher reaction temperature.

3.5. Thermal stability

Fig. 6 shows the thermal stability of the free and the immobilized lipases studied in this work. It was found that the free lipase lost all its initial activity within about 100 min. Generally speaking, the enhanced hydrophobic interaction could increase

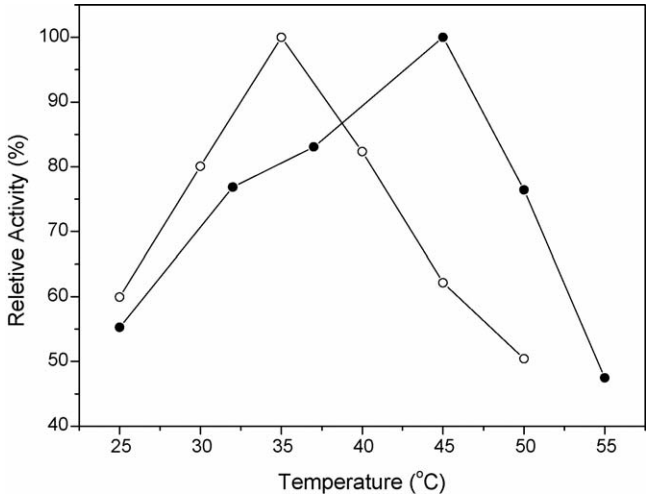


Fig. 5. Effect of temperature on the activity of lipase: (◯) free lipase; (●) adsorbed lipase on PSF/6 wt.% PVP nanofibers. Reaction pH 7.0.

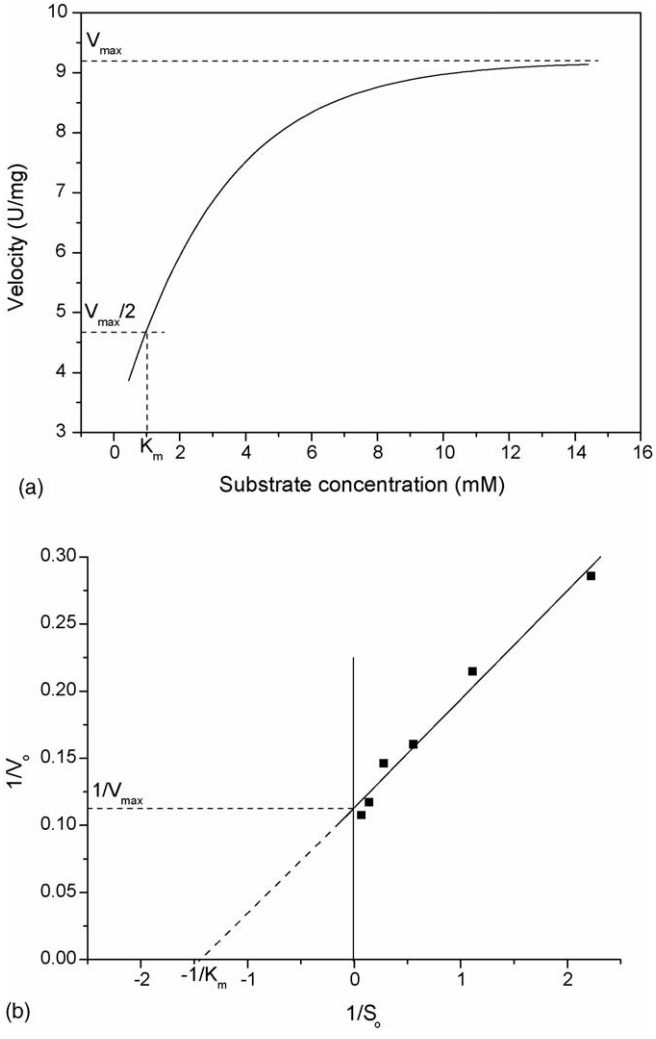


Fig. 7. Calculation of kinetic parameters for absorbed lipase: (a) Michaelis–Menten plot; (b) Lineweaver–Burk plot (double reciprocal plot).

the thermal stability; in other words, herein, the weakened surface hydrophobicity due to PVP aggregation was against the thermal stability of the absorbed lipase. However, the aggregated PVP might increase the surface biocompatibility, which could reasonably explain the enhanced thermal stability of the lipase immobilized on the PSF/PVP electrospun nanofibrous membranes.

3.6. Kinetic parameters

Kinetic parameters for the activity of free and immobilized lipase, V_{max} and K_m, were assayed at substrate concentration from 1 to 14.4 mM. V_{max}, which defines the highest possible velocity when all the enzyme is saturated with substrate, reflects the intrinsic characteristics of the immobilized enzyme, but may be affected by diffusion constrains. K_m, or apparent K_m, which is defined as the substrate concentration that gives a reaction velocity of 1/2 V_{max}, reflects the effective characteristics of the enzyme and depends upon both partition and diffusion effects [6] (Fig. 7a).

The kinetic parameters K_m and V_{max} were calculated from double reciprocal plot, as indicated in Fig. 7b (free lipase not indicated). K_m value was 0.45 mM for the free lipase, while the apparent value was 0.691 mM for the absorbed lipase, while the V_{max} value for the former preparation (46.4 U/mg) was found to be higher than that for the latter preparation (8.886 U/mg). This increase was either due to the conformational changes of the enzyme resulting in a lower possibility of forming a substrate-enzyme complex, or a less accessibility of the substrate to the active sites of the immobilized enzyme caused by the increased diffusion limitation.

4. Conclusion

Electrospun polysulfone nanofibrous membranes containing PVP or PEG as additives were applied to immobilize *C. rugosa* lipases by physical adsorption. The moderate adsorption time was determined as 90 min to make the lipase fully contact with the nanofibrous membranes. It was found that the post-treatment had little effect on the amount of enzyme loading. Increasing PVP or PEG content could enhance the adsorbed lipase activity due to the biocompatibility they rendered, although the amount of absorbed lipase decreased, which might be attributed to increased fiber diameter and weakened adsorption strength by fiber surface hydrophilicity. In addition, compared with free lipase, the optimum temperature for adsorbed lipase activity increased, pH value got lower and thermal stability increased. The kinetic parameters of the free and immobilized lipases, K_m and V_{max} were assayed. The V_{max} and K_m values for immobilized preparations were lower and higher those for free preparations. These results show that polysulfone nanofibers were potential support in the enzyme immobilization technology for industry applications and blending biopolymers into the nanofibers was a feasible method to improve enzyme activity, while the amount of bound enzyme decreased little.

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