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A comparative study on lipase immobilized polypropylene microfiltration membranes modified by sugar-containing polymer and polypeptide

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Abstract

Two protocols were used to modify polypropylene microfiltration membrane (PPMM): (a) a sugar-containing polymer was grafted onto the membrane surface by the plasma-induced polymerization of α-allyl glucoside (AG) and (b) poly(γ-stearyl L-glutamate) (PSLG) was tethered onto the membrane surface through the ring opening polymerization of *N*-carboxyanhydride (NCA) derived from γ-stearyl L-glutamate. Lipases from Candida rugosa were immobilized on these membranes by adsorption. Results on the basis of the enzyme adsorption capacity, activity and thermal stability were compared with those of the nascent PPMM. It was found that, as for the PAG-modified PPMM, the adsorption capacity and the activity retention of lipases were lower than those of the nascent ones, but the thermal stability was improved to some degree. On the other hand, tethering PSLG on the membrane surface increased the activity retention of lipases immobilized on the membrane from 57 to 72%, and the thermal stability was also improved.

Keywords: Lipase; Polypropylene microfiltration membrane; Enzyme immobilization; Sugar-containing polymer; Poly(γ-stearyl L-glutamate); Surface modification

1. Introduction

Lipases have gained considerable importance as versatile biocatalysts for the hydrolysis/synthesis of a wide range of esters and amides [1–3]. A promising property of lipases is their activation in the presence of hydrophobic interface, which was first reported by Sarada and Desnuelle [4]. Up to now, this lipase activation at interfaces has been recognized as a common feature. 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. In this case, lipases seem to become strongly adsorbed to hydrophobic interfaces through a large hydrophobic surface that surrounds the catalytic site. This large hydrophobic surface involves residues from the internal face of the lid as well as from other protein chains. Therefore, in recent years, special emphasis has been paid to the selective adsorption of lipases on tailor-made strongly hydrophobic support surfaces [5–13]. This immobilization procedure is based on the assumption that the large hydrophobic area that surrounds the active site of lipases is the one mainly involved in their adsorption on strongly hydrophobic solid surfaces. Thus, lipases recognize these surfaces similarly to those of their natural substrates and they suffer interfacial activation during immobilization. Main advantages for this immobilization method include: (a) promoting a dramatic activation of lipases after their immobilization (that is, adsorbed lipases show very enhanced esterase activity in the absence of additional hydrophobic interfaces); (b) promoting a possibility to associate the immobilization with the purification of lipases and (c) promoting a strong but reversible immobilization that enables us to recover these expensive supports after inactivation of immobilized lipases.

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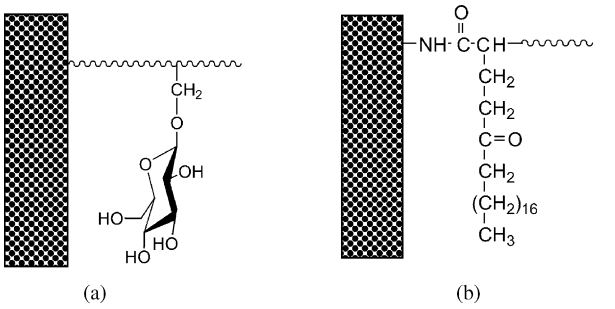

Fig. 1. Schematic representatives for the (a) PAG-modified PPMM and (b) PSLG-modified PPMM.

Artificial membranes have been applied in biotechnology, due to their interesting properties of high specific surface area and the possibility to combine separation with chemical reaction [14]. The fact that lipases are activated in the presence of aqueous/hydrophobic interfaces has interested membrane scientists and biotechnologists in their attempt to find polymeric membrane as an efficient carrier for the immobilization of enzyme. Various membrane materials from hydrophilics to hydrophobics were reported in the literature [12,13,15–26]. Among them, polypropylene membrane is more interesting [15–18] due to its hydrophobicity, well-controlled porosity, and chemical inertness as well as high potentials for comprehensive applications. In the present work, polypropylene microfiltration membrane (PPMM) which is in hollow fiber form was employed as the matrices for surface modification and lipases immobilization. A sugar-containing polymer was grafted onto the membrane surface by the plasma-induced polymerization of α-allyl glucoside (AG) [27] to create a hydrophilic and biocompatible interface for lipase immobilization (Fig. 1a). In addition, poly(γ-stearyl L-glutamate) (PSLG), a polypeptide with long hydrophobic side chain, was tethered onto the membrane surface through the ring opening polymerization of *N*-carboxyanhydride (NCA) of γ-stearyl L-glutamate initiated by amino groups (Fig. 1b) [28]. Then, lipases from Candida rugosa were adsorbed on these surface-modified membranes. The adsorption, activity and thermal stability of immobilized lipases were investigated. Since lipases possess an unusually high affinity to the surface of hydrophobic solids, a continuous study on finding suitable hydrophobic membranes as the carrier for the enzyme in biotransformation is needed.

2. Experimental

2.1. Materials

Polypropylene hollow fiber microfiltration membrane was prepared with melt-extruded/cold-stretched (MECS) method in our lab [27,28]. The inner and outer diameters of this hollow fiber are 240 and 290 μm, respectively, with porosity of 50% and an average pore diameter of 0.10 μm. Lipase (from Candida rugosa), Bradford reagent [29] and bovine serum albumin (BSA) were purchased from Sigma and used as received. α-allyl glucoside (AG) was synthesized with the method reported by Talley et al. [30]. The synthesis of γ-stearyl L-glutamate (SLG) and γ-stearyl L-glutamate NCA (SLGNCA) was referred to the method reported in our previous paper [28]. *N*-dimethyl formamide (DMF) is commercial product and was purified by vacuum distillation before used. Tetrahydrofuran (THF) was washed with stannous chloride solution, refluxed with sodium using benzophenone as indicators, and distilled before use. All other chemicals are of analytical grade and used without further purification.

2.2. Modification PPMM surface with poly(α-allyl glucoside)

Nascent PPMM was soaked in acetone for 2 h to remove any chemicals absorbed on the membrane surfaces. After this, the membrane was washed with acetone and dried in a vacuum oven at room temperature for 4 h. A two-stage procedure was used for membrane modification. First, PPMM was dipped into an AG solution of DMF for a certain time and then taken out to evaporate the solvent in vacuum oven. After that, the dip-coated AG was fixed chemically onto the membrane surface by N_2 plasma radiation. Plasma treatment was carried out in a plasma reactor. The reactor was connected with vacuum and diffusion pumps. Two electrodes were powered through a matching network by a 13.5 MHz radio-frequency generator. PPMM was placed in the middle of two electrodes of the reactor apparatus. The reactor was evacuated to 60 mTorr. Then, 100 W nitrogen plasma was used for various times. After this, the membrane was taken out of the reactor and washed with excess water and extracted in a Soxhlet extractor using acetone as solvent for 24 h to remove any residual monomer and AG homopolymer. Finally, the membrane was dried in a vacuum oven at 50 °C for 24 h.

2.3. Modification PPMM surface with poly(γ-stearyl L-glutamate)

PPMM was fixed in the plasma reactor. Pressure in the reactor was reduced and then NH_3 was introduced. This process was conducted several times to ensure that O_2 in the reactor was almost degassed. Then plasma was generated at a given pressure (5–60 Torr) and the PPMM was exposed to the NH_3 plasma for a predetermined period of time. After plasma treatment, the membrane was quickly dipped into a THF solution containing the SLGNCA. The graft polymerization was lasted for 3 days at 35 °C in N_2 atmosphere with constant vibration. Finally, methanol was added to terminate the polymerization, the resulted membrane was washed several times with THF, and dried at vacuum till constant weight.

2.4. Structure analysis and properties measurement for the modified membranes

Fourier transform infrared spectroscopy (Vector 22 FT-IR, Brucker Optics, Swiss) with an ATR unit (KRS-5 crystal, 45°) was used to investigate the chemical changes between the original PPMM and the modified ones. Spectra of X-ray photoelectronic spectroscopy (XPS) for the original and the modified membranes were also recorded with a PHI 5000C XPS spectrometer (Perkin Elmer Instruments, USA) using a monochromatic Al Kα X-ray source at a taking off angle of 45°. Water contact angle on the membrane surface was measured with a sessile drop method at 25 °C under an atmosphere of saturated water vapor by a KRUSS DSA10-MK machine. At least 10 contact angles were averaged to get reliable data.

2.5. Immobilization of lipase by adsorption

Lipase solutions (0.25–2.50 mg/ml) were prepared by adding appropriate amounts of lipase powder to phosphate buffer (0.05 M, pH 7.0). A bundle of hollow fiber membranes was immersed into ethanol for 1 h, thoroughly washed with deionized water to remove residual ethanol, and then rinsed with buffer. After this, the pretreated membranes were submerged into the lipase solution in a vertical orientation and shaken gently in a water bath at 30 °C for 3 h. Finally, the membranes were taken out and rinsed with buffer till no soluble protein was detectable in washings. Protein concentration in solutions were determined with Coomassie Brilliant Blue reagent following Bradford's method [29]. Bovine serum albumin was used as standard to construct the calibration curve. The amount of adsorbed protein on the membranes was calculated from the protein mass balance among the initial, final lipase solution and washings. The lipase adsorption capacity of the membranes was defined as the amount of protein (mg) per square meter of exposed surface areas of the hollow fiber membranes.

2.6. Desorption experiments

The lipase immobilized membranes were incubated in phosphate buffer (0.05 M, pH 7.0) and shaken in a water bath at 30 °C for 24 h. Supernatant samples were removed at intervals and protein content was analyzed as described above. The desorption ratios of lipases was calculated by the following expression:

$$\text{Desorption ratio} = \frac{\text{amount of lipases released} \times 100}{\text{amount of lipases adsorbed on the membranes}}$$

2.7. Activity assay of free and immobilized lipases

The pH stat method with olive oil titrimetric assay was used in this work. The lipase immobilized hollow fiber membranes were cut into short segments to make them disperse well in the emulsion during assay process. The substrate emulsion was prepared by thoroughly mixing 130 ml olive oil with 400 ml gum arabic solution (11% gum arabic powder and 1.25% $CaCl_2 \cdot 2H_2O$, m/v) and stored at 4 °C. Substrate emulsion of 24 ml was mixed with 9 ml deionized water and 2 ml sodium taurocholate solution (0.5%, m/v). The emulsion was incubated in a water bath at a certain temperature for several minutes, and then pH was adjusted to the desired value with NaOH solution. Lipase solution of 1 ml (1 mg/ml) or suitable amounts of the lipase immobilized membranes was added into the emulsion. The pH was held constant for 10 min by continuously adding 0.01 M NaOH standard solution. The consumed volume of NaOH standard solution was recorded. The blank value was measured by the same way.

One lipase unit corresponded to the release of 1 μmol of fatty acid per minute under the assay conditions. The enzyme activity was the number of lipase unit per square meter of exposed surface areas of the hollow fiber membranes. Specific activity was defined as the number of lipase unit per milligram of protein.

2.8. Thermal stability

Free and immobilized lipase preparations were stored in phosphate buffer (0.05 M, pH 7.0) at 50 °C for 2 h. Parts of them were periodically withdrawn for activity assay. The residual activities were determined as above.

3. Results and discussion

3.1. Characterization of the surface-modified membranes

The membrane surface of PPMM was modified by the graft polymerizations of α-allyl glucoside and γ-stearyl L-glutamate *N*-carboxyanhydride, respectively. To investigate the chemical changes between the original PPMM and the modified membranes and to confirm the poly(α-allyl glucoside) (PAG) and/or poly(γ-stearyl L-glutamate) (PSLG) formed on the membrane surface, FT-IR/ATR and XPS spectra were measured. There is one weak absorbance at 3000–3700 cm^{-1} in the FT-IR/ATR spectrum of the original PPMM. From the FT-IR/ATR spectra of PAG-modified membranes, one distinct absorption peak can be seen at 3350 cm^{-1} which is attributed to the hydroxyl groups of the glucoside moieties. After tethering poly(γ-stearyl L-glutamate) (PSLG) on the membrane surface, the FT-IR/ATR spectrum also shows that the weak absorbance at 3000–3700 cm^{-1} for original membrane improves greatly after modification, which implies the existence of $–NH_2$, –OH and hydrogen bond between them. In addition, the absorbance peak at 1640 cm^{-1} in the spectrum of PSLG-modified PPMM further indicates the existence of amide backbone. Furthermore, for unmodified PPMM, a major emission peak corresponding to 285.0 eV binding en-

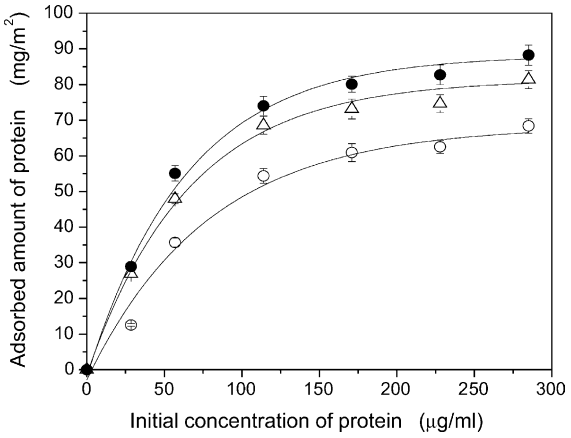

Fig. 2. Effect of initial protein concentration on the adsorbed amount of protein. (●) nascent PPMM; (○) 3.70 wt.% PAG-modified PPMM; (△) 3.6 wt.% PSLG-modified PPMM.

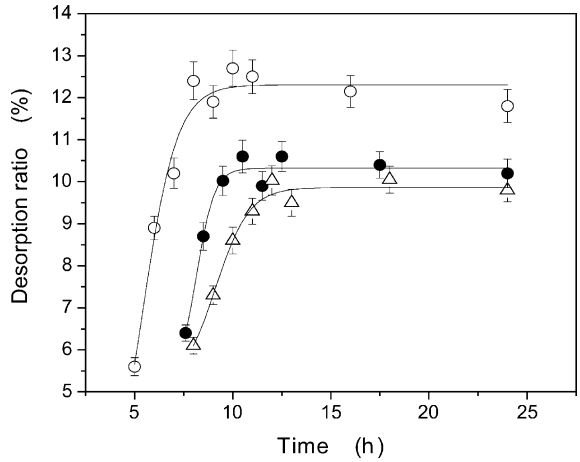

Fig. 3. Effect of time on desorption ratio. (●) nascent PPMM; (○) 3.70 wt.% PAG-modified PPMM; (△) 3.6 wt.% PSLG-modified PPMM.

ergy of C_{1S} can be observed in the XPS spectrum. However, for the PAG-modified membranes, an additional emission peak (533.8 eV binding energy) of O_{1S} can be seen, and the peak intensity for O_{1S} increases with the grafting degree of α-allyl glucoside. All these results demonstrate that the membrane surface of PPMM is successfully modified.

3.2. Adsorption/desorption behaviors of lipases

The effect of initial protein concentration on the adsorbed amount of protein is shown in Fig. 2. It can be seen that, for each membrane support, the increment of protein concentration enhances the driving force for the adsorption and increases the adsorbed amount of protein correspondingly. Furthermore, the results suggest that, at the same adsorption conditions, the adsorbed amount of protein on the membranes studied in this work decreases in the sequence of nascent PPMM > PSLG-modified PPMM > PAG-modified PPMM. The protein adsorbed on the PAG-modified PPMM is much lower than that on the nascent ones. This result might be due to the difference of adsorption strength. According to the complex multiple conformation properties of lipases, the interfacial hydrophobic interaction between the support surface and the hydrophobic domain around lipase's active center seems to dominate the adsorption strength [3,5–10]. As for the PAG-modified membrane, although the grafting degree of α-allyl glucoside is rather low (about 3.7 wt.%), the modifying process still results in high hydrophilicity on the membrane surfaces, which can weaken the hydrophobic interaction and reduce the adsorption capacity. On the other hand, several literatures described the secondary structure of poly(L-glutamates) [31–35]. Poche et al. [31] speculated that PSLG in solution forms an α-helix conformation which is stabilized by the intermolecular hydrogen bonds, with the peptide main chains in the cores and the γ-stearyl long side chains stretching outside. This molecular model of PSLG would effectively reinforce the hydrophobic interaction between the PSLG-modified PPMM surface and lipases. However, the results in this work suggest that the PSLG-modification protocol offers lower adsorption capacity compared with the nascent ones. It could be ascribed to the partial block of the membrane pores and the reduction of the available adsorption areas by the polypeptide chains on the membrane surface. On account of the influence of membrane pores, it is necessary to confirm further the driving forces for lipase adsorption by the desorption experiments. The plot of desorption ratio as a function of time is shown in Fig. 3. The data were obtained from the protein concentration in phosphate buffer after the lipase immobilized membranes were incubated in this buffer solution at 30 °C. In that case, only the lipases adsorbed weakly on the membranes can be desorbed. It was found that the time needed to reach about 6% desorption ratio is 5 h for the PAG-modified PPMM, while this period prolongs to about 8 h for the nascent PPMM and the PSLG-modified PPMM. Furthermore, the desorption ratio at desorption/adsorption equilibrium is about 12, 10 and 9.5%, respectively, for the PAG-modified PPMM, nascent PPMM and PSLG-modified PPMM, respectively. This sequence is consistent with the surface hydrophobicity of the membrane as PSLG-modified PPMM > nascent PPMM ≫ PAG-modified PPMM (Table 1). These results to some extent confirm the fact that interfacial activation on hydrophobic supports is the dominant adsorption strength for lipase adsorption in our case.

3.3. Effect of surface modification on the activity of lipases

Comparison for the adsorption capacity and the activity of lipases on the nascent, PAG- and PSLG-modified PPMMs is shown in Table 1. It can be seen that the introduction of PSLG on the membrane surface make the activity retention of lipases increases from 57.5 ± 2.8 to 72.4 ± 3.9%. This encouraging change seems due to the strong hydrophobic interaction between the long stearyl chains dispersing on the membrane surface and the hydrophobic domain around the

Table 1
Comparison of adsorption capacity and activity of lipases on nascent, PAG- and PSLG-modified PPMMs

Membrane	Grafting degree (wt.%)	Water contact angle (°)	Adsorbed protein (mg/m^2)	Specific activity (U/mg protein)	Activity retention (%)
Nascent PPMM	0	118	82.7 ± 2.6	69.9	57.5 ± 2.8
PAG-modified PPMM	3.7	36	62.5 ± 1.8	60.7	49.9 ± 2.9
PSLG-modified PPMM	3.6	122	74.6 ± 2.5	88.1	72.4 ± 3.9

Table 2
Effect of grafting degree on the adsorption and activity of lipases on PAG-modified PPMM

Grafting degree (%)	Water contact angle (°)	Adsorbed protein (mg/m^2)	Specific activity (U/mg protein)	Activity retention (%)
0	118	82.7 ± 2.8	69.9	57.5 ± 2.8
2.1	60	73.1 ± 2.8	61.3	50.4 ± 2.7
2.8	48	66.4 ± 2.7	62.3	51.2 ± 2.8
3.7	36	62.5 ± 1.8	60.7	49.9 ± 2.9

lipase' active site, which stabilize the 'open state' conformation of lipases and favor the active site's accessibility to substrates. On the other hand, after modification with PAG, the activity retention of lipase decreases from 57.5 ± 2.8 to 49.9 ± 2.9%. It could be reasonably explained as that the sharply hydrophilicity increase on the PAG-modified PPMM induce the conformational equilibrium of lipase to some extent shifts towards the unfavorable 'closed state'. Furthermore, in order to intensively understand the effect of surface hydrophilicity on the enzyme activity, water contact angles on the PAG-modified PPMMs with different grafting degree were measured. Results in Table 2 indicate that the water contact angle of PAG-modified PPMM decreases from 108° to 36° due to the grafting degree increasing from 0 to 3.7 wt.%. Nevertheless, the significant increment of hydrophilicity does not correspondingly result in the decreasing trend of activity retention. Similar results had also been observed by other researchers [6,7]. Typical explanation is that the chemical properties of the support surface directly affect the orientation of the enzyme active site upon adsorption. Therefore, in our situation, we might as well put forward a hypothesis that there is some affinity form of the glucoside moieties on the PAG-modified PPMM surface to lipase's hydrophilic areas far from active site like glycoprotein components. This particular affinity might offer a preferential orientation of lipase upon adsorption and somehow result in the activity's independence of support's hydrophilicity.

3.4. Thermal stability

Fig. 4 shows the thermal stability of the free and the membrane immobilized lipases studied in this work. It can be seen that the free lipase lost all its initial activity within about 100 min. Lipase adsorbed on the PSLG-modified PPMM preserve initial activity about 58% in 2 h. This value is 42% higher than that of the nascent PPMM. It seems that the stearyl chains on the membrane surface stabilize the conformation of enzyme protein and improve the resistance of protein to thermal denaturation. In addition, the PAG modification protocol also improves the thermal stability of the membrane immobilized lipases slightly (residual activity in 2 h: from 42 to 48%). It might be attributed to the stabilizing action on enzyme protein caused by some form of interaction between the glucoside moieties on membrane surface and the glycoprotein of Candida rugosa lipases.

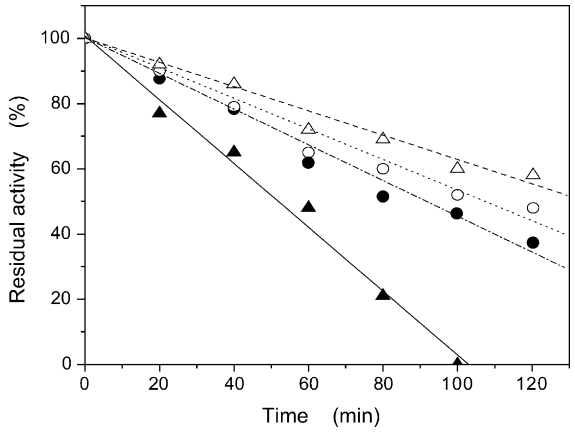

Fig. 4. Effect of time on residual activity. (▲) Free lipases; (●) nascent PPMM; (○) 3.70 wt.% PAG-modified PPMM; (△) 3.6 wt.% PSLG-modified PPMM.

4. Conclusions

Poly(α-allyl glucoside) (PAG) and poly(γ-stearyl L-glutamate) (PSLG) were respectively grafted onto the surface of polypropylene microfiltration membranes to create a biocompatible interface for lipase immobilization. Lipases from Candida rugosa were immobilized on the membrane surface by adsorption. For the PAG-modified PPMMs, the remarkable increase of hydrophilicity on the membrane surface after modification results in a slight increase of thermal stability but a minute decrease of activity retention for the enzyme. Both the activity retention and the thermal stability of lipase are improved by the modification on the membrane surface with PSLG. It might be mainly attributed

to the enhanced interfacial hydrophobic interaction between lipase and the support caused by the long stearyl chains of PSLG on the membrane surface.

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