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# Quantitative enzyme immobilization: Control of the carboxyl group density on support surface

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ABSTRACT

In this paper, carboxyl groups were quantitatively introduced onto a support surface by casting a polystyrene (PS) solution with small molecular weight α,ω-dicarboxyl-terminated polystyrene (PS-$(COOH)_2$) onto a glass plate. The support surface was examined using X-ray photoelectron spectroscopy (XPS) and contact angle measurements. The surface density of the carboxyl groups was measured by UV spectrophotometry with methylene blue (MB) dye as an indicator. Lipase (from *Candida rugosa*) as a model enzyme was covalently immobilized on the support surface in the presence of 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxylsuccinimide (NHS). It was found that with an increase of –COOH density from $0.42 \times 10^{-6}$ mol/m$^2$ to $1.21 \times 10^{-6}$ mol/m$^2$, the amount of immobilized protein increased linearly from 3.1 mg/m$^2$ to 4.1 mg/m$^2$, while the specific activity of lipase decreased from 7.79 U/mg to 5.14 U/mg with a breakpoint at the –COOH density of $1.08 \times 10^{-6}$ mol/m$^2$. As the trend of the specific activity of immobilized lipase was opposite to that of the amount of immobilized protein, the optimum carboxyl group density for obtaining the highest enzyme activity (26.4 U/m$^2$) was $1.08 \times 10^{-6}$ mol/m$^2$ and the molar ratio of –COOH/lipase was 16.2. The decreasing rate of the specific activity of the immobilized enzyme accelerated when –COOH density was above $1.08 \times 10^{-6}$ mol/m$^2$, which may be regarded as a critical value of –COOH density, leading to the enzyme immobilization changing from single-site to multi-site immobilization. After immobilization, the optimum pH of enzyme was broader and the optimum temperature of enzyme was enhanced to 50 °C. This work is helpful for the design and preparation of supports for the immobilization of enzymes of higher activity and for increasing the understanding of the relationship between conformation variation of an immobilized enzyme and its corresponding catalytic activity.



## 1. Introduction

Compared with conventional chemical catalysts, enzyme as biocatalysts exhibit a high level of catalytic efficiency and high degree of specificity, which enable them to catalyze a series of biotransformation reactions, yielding a wide range of useful biological and chemical compounds [1,2]. A number of problems exist in the practical application of enzymes, such as high cost and the instability of their structures. To efficiently exploit the advantages of enzymes, it is recommended to use them in an immobilized state to reduce the cost and generally enhance their stability by proper design [3–5]. Immobilization also facilitates the separation of products and provides more flexibility with enzyme/substrate contact by using various reactor configurations [6–9].

The properties of the support surface play a vital role in enzyme immobilization since there are various interactions formed between the support surface and an immobilized enzyme, including hydrophobic interaction, electrostatic interaction and chemical bonding. Thus, considerable effort has been made to tailor the properties of the support surface, such as introducing functional groups onto the support surface to enhance the hydrophilicity of the support surface [10–12] or creating a bio-mimetic microenvironment on the support surface [8,13,14].

The carboxyl group, a typical functional group, has been widely studied as an active site for enzyme immobilization. Most studies have focused on methods to introduce carboxyl groups onto the support surface. Yao and coworkers immobilized α-chymotrypsin onto carboxyl-functionalized superparamagnetic nanogels, prepared via *in situ* copolymerization of methylacrylic acid and methylene-bis-acrylamide [15]. Steffens et al. used poly (D,L-lactide) grafted with polyacrylic acid to immobilize horseradish peroxidase [16]. Due to the high carboxyl group density on the support surface, a high enzyme binding was

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readily obtained with a low activity of only 1%. Kawaguchi and coworkers prepared a thermosensitive hydrogel microsphere by precipitation polymerization of N-isopropylacrylamide, acrylamide, and methylene-bis-acrylamide [17]. Carboxyl groups on the microspheres were introduced by hydrolysis, and trypsin was immobilized on the carboxylated microspheres using carbodiimide. Kang and coworkers covalently immobilized glucose oxidase (GOD) on electroactive polyaniline (PAN) films with surface-grafted acrylic acid (AAc) polymer in the presence of a water-soluble carbodiimide [18].

However, there remains a lack of quantitative investigations concerning the relationship between carboxyl group density on the support surface and enzyme immobilization. Such information is meaningful for application of immobilized enzyme catalysts, since both the conformation and the catalytic environment of an enzyme are changed after enzyme immobilization, resulting in a noted decrease in enzyme activity. Therefore, it remains a challenge to clarify the effect of the chemical composition of the support surface on the enzyme conformation and activity. For this purpose, it is necessary to quantitatively control the kind and number of functional groups on the support surface. Consequently, the interaction between the support surface and the immobilized enzyme can be controlled more precisely and studied in greater detail.

The modification of polymer end groups has emerged as a practical means of controlling polymer surface properties [19–21]. In our previous work [22], polystyrene (PS) membrane surfaces with carboxyl were prepared by casting α,ω-dicarboxyl-polystyrene (PS-$(COOH)_2$) solution onto a glass plate. Carboxyl end groups are localized at the glass-side surface of a PS membrane in order to minimize the interfacial free energy between the membrane and the glass plate, as documented by XPS analysis, surface energy and contact angle measurements. In the present study, carboxyl groups were quantitatively introduced onto a support surface by casting PS solutions with small molecular weight PS-$(COOH)_2$ onto a glass plate. Lipase, with the open and closed conformations, is one of the most utilized enzymes [23,24] and various immobilization methods were reported, such as lipase adsorption on hydrophobic supports or immobilization in the presence of detergents [25,26]. In our experiments, lipase (from *Candida rugosa*) was used as a model enzyme to be covalently immobilized on the support. The effect of –COOH density on support surface on the activity, catalytic properties, thermal stabilities and kinetic parameters of the immobilized enzyme were investigated.

## 2. Materials and methods

### 2.1. Materials

Lipase (from *C. rugosa*), Bradford reagent, bovine serum albumin (BSA), 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxyl succinimide (NHS), p-nitrophenyl palmitate (p-NPP) and 2-morpholino-ethane sulfonic acid (MES) were purchased from Sigma and used as received. Maleic anhydride was purchased from Aldrich Co. and was recrystallized before use. Polystyrene (Mn 92,100, PDI 2.6) was purchased from Aldrich Co. α,ω-dicarboxyl-terminated polystyrene (PS-$(COOH)_2$) (Mn 4400, PDI 2.0) was prepared as previously reported [22]. All other chemicals were of analytical grade and used without further purification.

### 2.2. Preparation of PS support with various –COOH density on its surface

Prescribed amounts of polystyrene were dissolved in xylene at a concentration of 5 wt% for the preparation of casting solutions. The PS films with –COOH groups were prepared by pouring PS casting solution with a small amount of PS-$(COOH)_2$ on a rimmed glass plate and drying at 25 °C in dried air, then drying at 150 °C for 10 h. The surface density of the carboxyl groups was quantitatively adjusted by changing the PS-$(COOH)_2$ content. Each film taken off the glass plate had two surfaces that were formed under different environments. One surface of the film was formed in contact with the dried air, and another surface was formed in contact with the glass plate. In this paper, we designate them the air-side surface and the glass-side surface of the film, respectively.

### 2.3. Characterization of the support surface

XPS experiments were performed on a PHI-5000C ESCA system (PerkinElmer) with Al Kα radiation ($h\nu$ = 1486.6 eV). Contact angles of water on the PS surfaces of the supports were measured by the Sessile drop method using a DSA-10 drop sharp analysis (Kruss Co., Germany) at 25 °C. The volume of the water drops used was always 3 μL. All reported values are averages of at least eight measurements taken at different locations on the film surface and have a typical error of the mean of ±1°. The morphologies of the supports were examined under a field emission scanning electron microscope (Hitachi, S-4800, Japan).

Load and release of methylene blue (MB): the support was submerged in a buffered MB solution at pH 7.0 with a concentration of MB of 10–3 M. Note that at neutral pH values, the carboxyl group is negatively charged and can form electrostatic interactions with the positively charged MB (loading step). After 2 h, the films were rinsed in a separate bath at pH 7.0 for 30 min, thereby removing the excess MB. Finally, the release of the MB was carried out by dipping the support into an acidic water solution (pH 3.0). The quantity of MB released was estimated by UV spectrophotometry (UV-2501PC, Shimadzu Co., Japan) [27].

### 2.4. Lipase immobilization

Lipase was immobilized onto the PS film support by an EDC/NHS activation procedure. An appropriate amount of the support was thoroughly washed with de-ionized water, and then rinsed with MES buffer (50 mM, pH 6.0). After this, the pretreated support was submerged in the EDC/NHS solution (20 mg/mL in MES buffer, 50 mM, pH 6.0, the molar ratio of EDC to NHS = 1:1) and shaken gently for 6 h at room temperature. The activated supports were removed, washed several times with phosphate buffered solution (PBS) (50 mM, pH 5.5) and submerged in the enzyme solution (2 mg/mL in PBS, pH 5.5). The immobilization process was carried out at room temperature in a shaking water bath for 8 h. Finally, the supports were removed, thoroughly rinsed with PBS (50 mM, pH 5.5) and then rinsed with de-ionized water. The amount of immobilized protein on the support was determined by measuring the initial and final concentrations of protein within the enzyme solutions and washings using Coomassie Brilliant Blue reagent, following Bradford's method [28]. BSA was used as the standard to construct a calibration curve. The immobilization capacity of the protein on the support was defined as the amount of protein per square meter of the support.

### 2.5. Activity assay of lipase

The reaction rate of the free and immobilized lipase preparations was determined according to the method reported by Chiou and Wu with only minor modifications [29]. In the standard conditions, the reaction mixture was composed of 1.0 mL ethanol containing 14.4 mM p-NPP and 1.0 mL PBS (50 mM, pH 7.5) in an Erlenmeyer flask. The reaction was initiated by addition of 0.10 mL free lipase preparation (or 100 mg immobilized lipase preparation). The reaction mixture was then incubated at 37 °C under reciprocal agitation

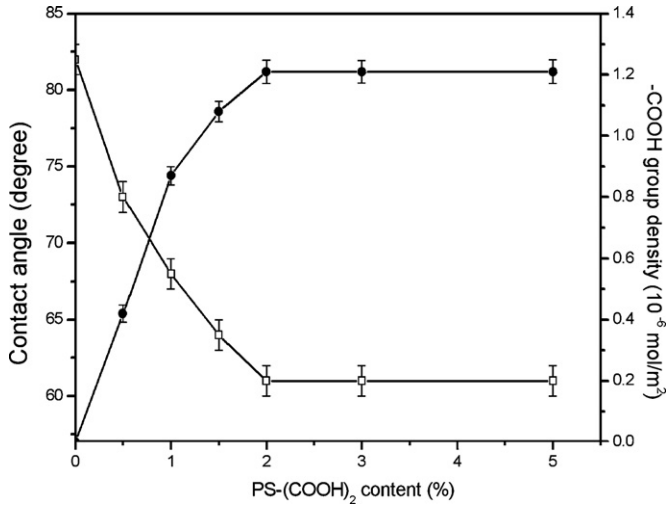


**Fig. 1.** Effect of PS-$(COOH)_2$ concentration on the contact angle of water (□) and –COOH density (●) on the glass-side surface of the support.

at 120 strokes per minute. After 5 min of reaction, agitation was stopped, and the reaction was then 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 volume was diluted 10-fold with de-ionized water, and measured at 410 nm with a UV–vis spectrophotometer (UV-1601, Shimadzu, Japan) against a blank without enzyme and treated in parallel. The reaction rate was calculated from the slope of the absorbance versus time curve. The molar extinction coefficient of $14.1 \times 10^3\ M^{-1}\ cm^{-1}$ for p-nitrophenol (p-NP), which was determined from the absorbance of standard solutions of p-NP in the reaction medium, was used. One enzyme unit is defined as the amount of biocatalyst liberating 1.0 μmol of p-NP per minute in these conditions. Enzyme activity is defined as the number of lipase units per square meter of support. Specific activity is defined as the number of enzyme units per milligram of protein. Activity retention is defined as the ratio of the activity of the amount of the enzyme coupled on the support to the activity of the same amount of free enzyme.

### 2.6. Thermal stability measurements

The thermal stabilities of the free and immobilized lipases were assessed by immersing them in PBS (50 mM, pH 7.0) for 2 h at 50 °C and periodically determining their activities.

## 3. Results and discussion

### 3.1. Characterization of the support surface with varying –COOH group density

Contact angle measurement is one of the most sensitive and effective methods for probing surface structure of polymers with 0.5–1 nm of surface sensitivity [30]. The contact angle of water on the glass-side surface of the support containing various PS-$(COOH)_2$ content was determined and the results are shown in Fig. 1. The contact angle of water on the air-side surface of the support dropped quickly from 82° to 61° as PS-$(COOH)_2$ content increased, and then remained constant after the PS-$(COOH)_2$ content was more than 2%. Conversely, the air-side contact angles were basically stable, essentially remaining constant at about 91°.

Further characterization of the surface chemistry of the carboxylated support was conducted by XPS analysis. The results in Table 1 show that the molar ratios of O/C on the glass-side surface gradually increased from 0.012 to 0.165 with the increase of PS-$(COOH)_2$ content from 0% to 2%, while those on air-side surface were relatively low and almost constant (about 0.01). Fig. 2 shows the high-resolution XPS spectra of the C 1s region for the air-side and glass-side surfaces of the support containing 1.5% PS-$(COOH)_2$. Four components, at 285.0 eV, 287.2 eV, 289.4 eV and 291.5 eV, were observed in the XPS spectra of the glass-side support surface, which correspond to neutral carbon, carbonyl group carbon, carboxyl group carbon and aromatic $\pi$–$\pi^*$ transition carbon, respectively [31]. Compared to the air-side surface, the appearance of the carboxyl group carbon peak was apparent in the XPS C 1s spectra of the glass-side surface. This suggests that the amount of –COOH groups was enhanced on the glass-side surface due to carboxyl group segregation.

**Table 1**
Surface element composition of the glass-side surface of the support.

| Content of PS-$(COOH)_2$ (%) | O (%) | C (%) | O/C |
|---|---|---|---|
| 0 | 1.2 | 98.8 | 0.012 |
| 0.5 | 8.1 | 91.9 | 0.088 |
| 1 | 11.4 | 88.6 | 0.129 |
| 1.5 | 13.1 | 86.9 | 0.151 |
| 2 | 14.2 | 85.8 | 0.165 |

The carboxyl group density on the support surface was measured by UV spectrophotometry with MB as indicator. Bousquet and

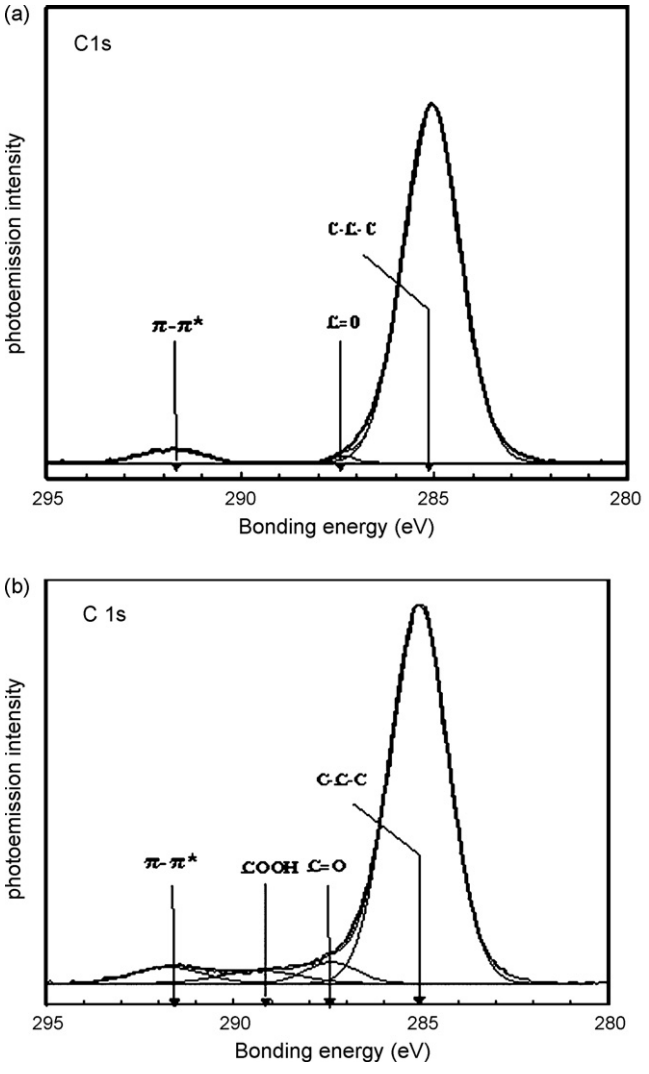


**Fig. 2.** High-resolution XPS spectra of C 1s region for the air-side (a) and glass-side (b) surface of the support containing 1.5% PS-$(COOH)_2$.

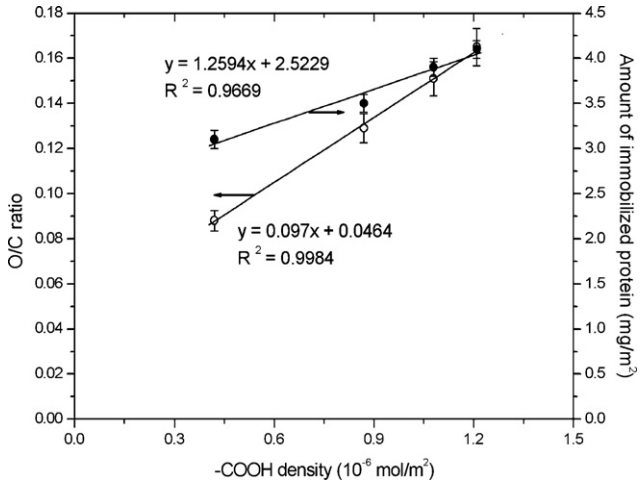


**Fig. 3.** Relationship between –COOH density on the support surface and O/C ratio determined by XPS (○), and the amount of immobilized lipase (●), respectively.

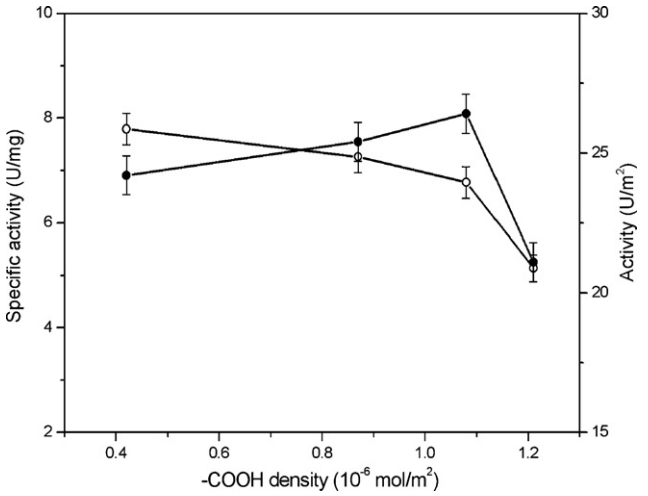


**Fig. 4.** Effect of –COOH density on the specific activity (○) and activity (●) of immobilized enzyme. The specific activity of free lipase: 40.8 U/mg.

Ibarboure used this method to measure the carboxyl group density on polymer surfaces [27]. This method uses a positively charged dye (MB) capable of establishing electrostatic interactions with the carboxyl group, which is anionic in neutral aqueous solution. When the pH is changed to acidic values, the carboxylic functional groups become protonated, and MB is thus released. The quantity of MB released at low pH values is therefore proportional to the accessible carboxylic groups on the surface and can be easily evaluated from the corresponding UV–vis spectra using the Beer–Lambert law. Fig. 1 shows that the carboxyl group density on the support surface increased from $0.42 \times 10^{-6}$ mol/m$^2$ to $1.21 \times 10^{-6}$ mol/m$^2$ as the PS-$(COOH)_2$ content increased, and then remained constant after the PS-$(COOH)_2$ content was more than 2%, which corresponded to the variation of contact angle. Additionally, there is a linear relationship between the O/C ratio from XPS analysis and the –COOH density obtained by the MB method, as evident in Fig. 3. This result further indicates that the decrease of contact angle with the increase of PS-$(COOH)_2$ content may be attributed to enhanced –COOH segregation on the glass-side surface of the support. Similar results were also found when adding a small amount of hydrophilic PDMS-b-PDEAA to modify the surface of poly(dimethylsiloxane) membranes [32]. According to the results above, carboxyl groups were quantitatively introduced onto the support surface and this support was accordingly employed to immobilize an enzyme in the experiments described in the next sections.

### 3.2. Effect of enzyme immobilization on the activity of lipase

The effect of –COOH density on the support surface on the amount of immobilized protein is shown in Fig. 3. The amount of immobilized lipase increased linearly from 3.1 mg/m$^2$ to 4.1 mg/m$^2$ with increasing –COOH density. Kang and coworkers also reported that the amount of immobilized GOD increased linearly with the concentration of surface-grafted acrylic acid (AAc) polymer chains [18]. In our experiments, the –COOH density was relatively low, so that the amount of immobilized protein was also low and consequently there were some areas of the support surface which were not covered by the enzyme. Thus, the amount of immobilized protein increased linearly over the entire range of –COOH densities. For the immobilization activated with EDC/NHS, it was usually reported that amide bond was formed. In fact, several side reactions also existed such as the rearrangement to *N*-acylurea and the hydrolysis to –COOH group. Those –COOH groups might form some ionic interactions with lipase and have some effect on the enzyme activity.

Fig. 4 shows the effect of –COOH density on the specific activity and activity of immobilized enzyme. It was found that the specific activity of immobilized lipase decreased with increasing –COOH density. Interestingly, a break point exists at $1.08 \times 10^{-6}$ mol/m$^2$ in Fig. 4. The specific activity of immobilized enzyme decreased gradually with increasing –COOH density from $0.42 \times 10^{-6}$ mol/m$^2$ to $1.08 \times 10^{-6}$ mol/m$^2$, while after this break point, over the range of –COOH density from $1.08 \times 10^{-6}$ mol/m$^2$ to $1.21 \times 10^{-6}$ mol/m$^2$, the decreasing rate of the specific activity accelerated. At the same time, the amount of immobilized protein increased linearly over the entire range of –COOH density (from Fig. 3). Thus, there appears to exist an optimum –COOH density at which immobilized lipase possessed the highest activity. In our experiments, the optimum density of –COOH at which the immobilized lipase appeared to have the highest activity (26.4 U/m$^2$) was $1.08 \times 10^{-6}$ mol/m$^2$.

A decrease of enzyme activity commonly occurs after immobilization, which may be attributed to a minor modification in the enzyme conformation that may lead to the distortion of amino acid residues involved in catalysis. The presence of random immobilization causes the substrate approach to the active site of the enzyme to become hindered, resulting in limitations imposed by slow mass transfer of substrate or product to or from the active site of the enzyme. In this experiment, the breakpoint may be attributed to the fact that the variation of –COOH density on the support surface results in the existence of different proportions of multi-site and single-site immobilized lipases. Multi-site immobilization means that one enzyme molecule is attached to the support surface by more than one chemical bond, which might largely inhibits enzyme conformation and reduces enzyme activity. In some cases, multi-site immobilization did not produce severe drops in enzyme activity, especially for glyoxyl and epoxy supports [33,34]. In this work, it could be found that the specific activity of enzyme decreased with the increase of carboxyl group density, so it might be reasonable to deduce that the increase of carboxyl group density lead to more multi-site immobilization and the multi-site immobilization depressed the specific activity of enzyme. Single-site immobilization forms via only one chemical bond between enzyme and support surface, which is favorable for maintaining enzyme activity [16,35].

When the –COOH density was $0.42 \times 10^{-6}$ mol/m$^2$, the amount of immobilized protein was 3.1 mg/m$^2$. Thus, the density of immobilized lipase (molecular weight: 62,000) on the support surface was $0.50 \times 10^{-7}$ mol/m$^2$ and the molar ratio of –COOH/lipase was 8.4, which led to a lower proportion of multi-site immobilized

**Table 2**
Kinetic parameters and activity retention of the free and immobilized lipases.

| –COOH density ($\times 10^{-6}$ mol/m$^2$) | Activity retention (%) | $K_m$ (mM) | $V_{max}$ (U/mg) |
|---|---|---|---|
| $0.42 \pm 0.03$ | $19.1 \pm 0.7$ | 1.12 | 8.76 |
| $0.87 \pm 0.03$ | $17.8 \pm 0.7$ | 1.25 | 8.22 |
| $1.08 \pm 0.03$ | $16.6 \pm 0.7$ | 1.36 | 7.66 |
| $1.21 \pm 0.03$ | $12.6 \pm 0.6$ | 1.45 | 5.71 |
| Free lipase | 100 | 0.42 | 45.6 |

lipase and an higher activity retention (19.1%). With the increase of –COOH density, the possibility of forming chemical bonds between lipase and the support surface also increased. Therefore, both the proportion of the multi-site immobilization and the amount of immobilized protein increased. When the –COOH density was $1.21 \times 10^{-6}$ mol/m$^2$, the amount of immobilized protein was 4.1 mg/m$^2$, the density of immobilized lipase on the support surface was $0.66 \times 10^{-7}$ mol/m$^2$ and the molar ratio of –COOH/lipase was 18.3, which led to a higher proportion of lipase multi-site immobilization and a lower activity retention (12.6%). In fact, each immobilized enzyme molecule covered a certain area of the support surface, which could contain relatively few or perhaps many –COOH groups. Therefore, there may be a critical value of –COOH density, at which the enzyme switched from single-site immobilization to multi-site immobilization. According to the accelerated decrease of the specific activity of the immobilized enzyme evident in Fig. 4, it appears that $1.08 \times 10^{-6}$ mol/m$^2$ may be the critical value in the present study, at which the molar ratio of –COOH/lipase was 17.1. A further investigation regarding the effect of –COOH density on the conformation of immobilized lipase will be undertaken in the future.

Additionally, it should be noted that the molar ratio of functional group/enzyme could be greater than one even for the single-site immobilization. In aqueous solution, the shape of an enzyme molecule appears to be round or clavated, with some functional groups located on its surface. Enzyme molecules with irregular shapes cannot form a completely compact layer on the support surface; as a result, there should be some area of the support surface which is not be covered by the enzyme molecule. Conversely, the functional groups on the enzyme surface cannot completely interact with the functional groups in the area of the support surface covered by the enzyme molecule. Therefore there should be some unreacted functional groups in the area of the support surface covered by the enzyme molecule.

As discussed above, certain areas of the support surface are not covered by the enzyme molecule. Therefore, the molar ratio of functional group/enzyme should be corrected to reduce the effect of the functional group in the area not covered by the enzyme. Lipase from *C. rugosa* appears its shape (6.49 nm × 9.75 nm × 17.56 nm) [36]. As the amount of immobilized enzyme was 4.1 mg/m$^2$, the area occupied by the enzyme should be 4.6 m$^2$ in theory, which is larger than the surface area. Since the surface of the polymer support cannot be absolutely flat, the real surface area is greater than the macro surface area. Scanning electronic microscopy (SEM) was employed to characterize the immobilized enzyme, given that immobilized lipase occupied near 1/3 surface area of the support (PS-COOH/PS 5%) and the diameter of lipase was about 10–20 nm. It is reasonable to assume that each immobilized enzyme molecule covers the same area. The ratio can be corrected using the following equation:

$$R_{F/E} = \frac{1}{3} R^*_{F/E} \left( \frac{A}{A_{max}} \right) \quad (1)$$

where $R_{F/E}$ is the molar ratio of functional group/enzyme after correction, $R^*_{F/E}$ is the molar ratio of functional group/enzyme before correction, $A$ is the amount of immobilized protein, $A_{max}$ is the maximum amount of immobilized protein (4.1 mg/m$^2$ on PS-COOH/PS 5%) and 1/3 is the ratio of the surface area of the support (PS-COOH/PS 5%) occupied by immobilized lipase. Thus, the value of the ratio of function group/enzyme was corrected from 8.4, 17.1 and 18.3 to 2.1, 5.3 and 6.1 for the support with –COOH density of $0.42 \times 10^{-6}$ mol/m$^2$, $1.08 \times 10^{-6}$ mol/m$^2$ and $1.21 \times 10^{-6}$ mol/m$^2$, respectively.

The kinetic parameters ($K_m$ and $V_{max}$) and activity retention of the free and immobilized lipases are listed in Table 2. Compared to the free enzyme, there was an obvious increase in $K_m$ values for the immobilized enzymes. $K_m$ is defined as the substrate concentration that gives a reaction velocity of $1/2V_{max}$. This parameter reflects the effective characteristics of the enzyme and depends upon diffusional effects. The increase in $K_m$ values was either due to the conformational changes of the enzyme, resulting in a lower possibility to form a substrate–enzyme complex, or to the lower accessibility of the substrate to the active sites of the immobilized enzyme caused by the increased diffusion limitation. With the increase of –COOH density, the $K_m$ value for the immobilized lipase increased. Thus, compared to the immobilized lipase on the higher –COOH density support, it was easier for the lipase immobilized on the lower –COOH density support to form the substrate–enzyme complex.

$V_{max}$ defines the highest possible reaction velocity when the enzymes are saturated with substrate; therefore, this parameter reflects the intrinsic characteristics of the enzyme, and it is only affected by diffusional constrains if the enzyme has a $K_m$ value that is higher than the diffusion under these conditions. As shown in Table 2, $V_{max}$ values demonstrated a decrease upon immobilization. The $V_{max}$ value of the immobilized lipase on the lower –COOH density support was higher than that on the higher –COOH density support, which corresponded to the specific activity and activity retention of the immobilized lipases, and the same reasoning could be used to explain this phenomenon.

### 3.3. Effect of pH and temperature on the lipase activity

The effect of pH on the activity of the free and immobilized lipases in the pH range of 4.0–8.0 at 37 °C is given in Fig. 5. For the free lipase the maximum activity was at pH 7.5, while it occurred in the pH range of 6.5–8.0 for the immobilized lipases. The optimum pH ranges of the immobilized lipases were broader than for the free one, which could be attributed to the formation of covalent bonds, which may limit the transition of enzyme conformation with the change of pH [35]. Therefore, the immobilized lipase samples maintained a relatively high activity over a broader pH range.

The effect of temperature on the activities of the free and immobilized lipase was examined at a pH of 7.5 over the temperature range of 20–55 °C (Fig. 6). For the free lipase, the optimum temperature was approximately 37 °C, while it shifted nearly to 50 °C for the immobilized lipases. This shift in optimum temperature for maximum activity may be due to either the creation of conformational limitations on the lipase movement as a result of the formation of covalent bonds between the enzyme and the support, or to a low restriction in the diffusion of the substrate at higher temperatures. Therefore, the immobilized lipases exhibited their catalytic activity at a higher reaction temperature.

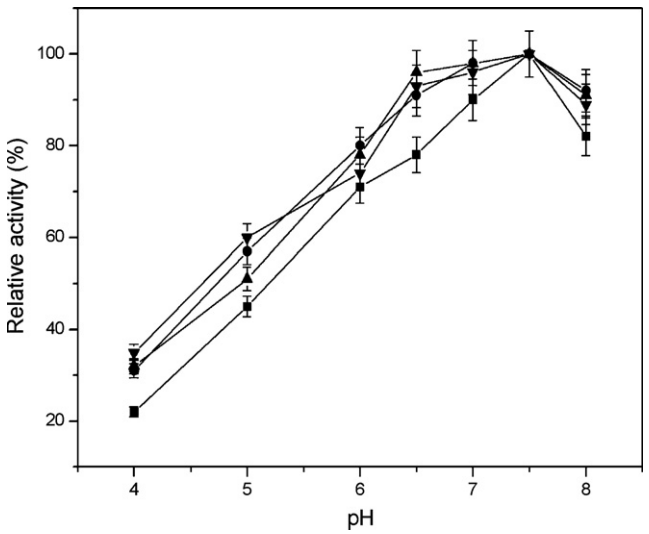


**Fig. 5.** Effect of pH on the activity of free and immobilized lipases at 37 °C. Free lipase (■); immobilized lipases on the support with –COOH density of $0.42 \times 10^{-6}$ mol/m$^2$ (●), $1.08 \times 10^{-6}$ mol/m$^2$ (▲) and $1.21 \times 10^{-6}$ mol/m$^2$ (▼).

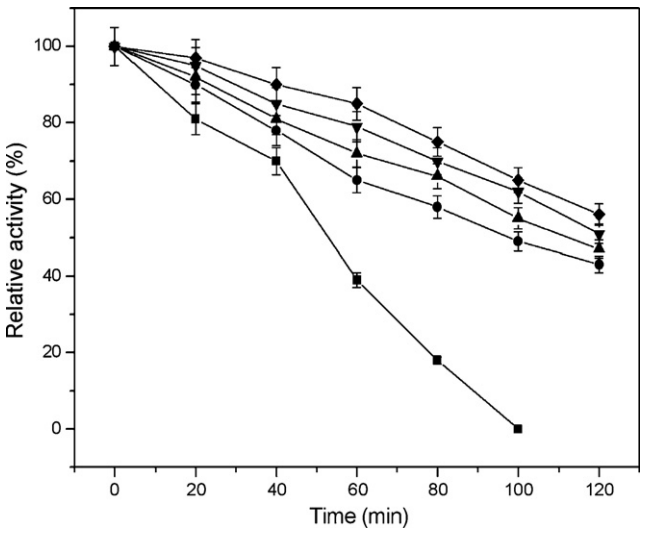


**Fig. 7.** Thermal stability of free and immobilized lipases. Free lipase (■); immobilized lipases on PS support with –COOH density $0.42 \times 10^{-6}$ mol/m$^2$ (●), $0.87 \times 10^{-6}$ mol/m$^2$ (▲) and $1.08 \times 10^{-6}$ mol/m$^2$ (▼) and $1.21 \times 10^{-6}$ mol/m$^2$ (♦).

### 3.4. Thermal stability

The thermal stabilities of the free and immobilized lipases are shown in Fig. 7. It can be observed that the free lipase lost its initial activity within 100 min at 50 °C, while the immobilized lipases retained their initial activities (which could be defined as the ratio of the enzyme activity after heat treatment to that before heat treatment) of about 43% (–COOH density $0.42 \times 10^{-6}$ mol/m$^2$), 47% (–COOH density $0.87 \times 10^{-6}$ mol/m$^2$), 51% (–COOH density $1.08 \times 10^{-6}$ mol/m$^2$) and 56% (–COOH density $1.21 \times 10^{-6}$ mol/m$^2$) after 120 min of heat treatment at 50 °C, respectively. These results indicate that the thermal stability of the immobilized lipases was much better than that of the free lipase. The rigid support (which does not swell in aqueous solution) with carboxyl groups on its surface fixed the relative positions of the groups implied in the immobilization, increasing the rigidity of the enzyme [37]. Thus, the interaction between the enzyme and the support could prevent the excessive conformation distortion of the enzyme at higher temperatures [38,39]. In addition, with increasing –COOH density on the support surface, the thermal stability of the immobilized lipases was enhanced. Such an enhancement of thermal stability may be attributed to the formation of multi-site enzyme immobilization which could more effectively confer rigidity to the enzyme and prohibit its conformation transition [3,4], thereby maintaining higher enzyme activity.

As discussed above, with increasing –COOH density, the specific activity of immobilized enzyme decreased, due to the increased proportion of the multi-site immobilized enzymes. At the same time, the thermal stability of immobilized enzyme increased with increasing –COOH density. Thus, there should also be an optimum –COOH density at which the immobilized enzyme with higher activity and thermal stability (which could be regarded as a typical stability) can be obtained. Knowledge of such an optimal –COOH density may be beneficial for applications of the immobilized enzyme, especially under severe reaction conditions or for prolonged periods of usage.

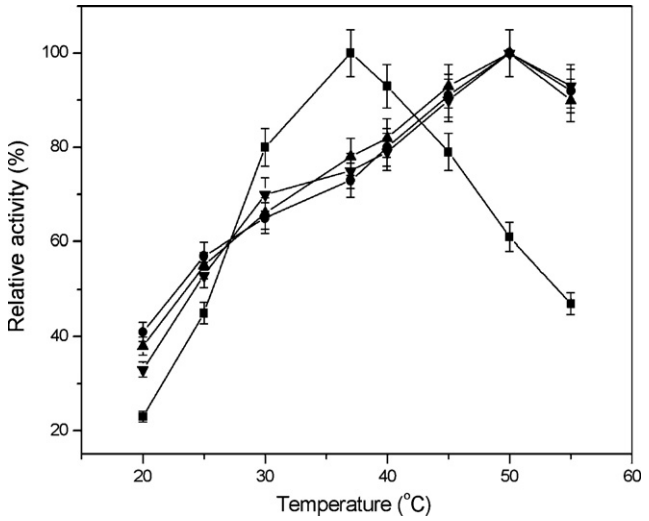


**Fig. 6.** Effect of temperature on the activity of free and immobilized lipases at pH 7.5. Free lipase (■); immobilized lipases on the support with –COOH density of $0.42 \times 10^{-6}$ mol/m$^2$ (●), $1.08 \times 10^{-6}$ mol/m$^2$ (▲) and $1.21 \times 10^{-6}$ mol/m$^2$ (▼).

## 4. Conclusion

A key factor for obtaining the highest activity of an immobilized enzyme is controlling the interaction between the immobilized enzyme and the support surface. In the present work, carboxyl groups were quantitatively introduced onto a support surface, and lipase as a model enzyme was then covalently immobilized on this support surface. It was found that with increasing –COOH density, the amount of immobilized protein increased linearly, while the specific activity of lipase decreased, with the existence of a breakpoint in the specific activity. At a –COOH density of $1.08 \times 10^{-6}$ mol/m$^2$, the immobilized lipase exhibited the highest activity (26.4 U/m$^2$). The decreasing rate of the specific activity of the immobilized enzyme accelerated during the range of –COOH density from $1.08 \times 10^{-6}$ mol/m$^2$ to $1.21 \times 10^{-6}$ mol/m$^2$. The value of $1.08 \times 10^{-6}$ mol/m$^2$ may also be a critical value of –COOH density at which the enzyme switched from single-site immobilization to multi-site immobilization. An investigation regarding the effect of –COOH density on the conformation of immobilized lipase will be carried out in future work. Conversely, with increasing –COOH density, the thermal stability of the immobilized lipase was also gradually enhanced, which has important implications for the applications of immobilized enzymes.

The present work may be useful for the design and preparation of supports for enzyme immobilization with higher activity and for increasing the understanding of the relationship between conformation variation of an immobilized enzyme and its corresponding catalytic activity. Moreover, this method may also be employed to quantitatively study the interaction between the support surface and other bio-macromolecules, such as nucleic acids and antibodies.

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