Influence of Varying Surface Hydrophobicity of Chitosan Membranes on the Adsorption and Activity of Lipase

Hong-Tao Deng, Juan-Juan Wang, Zhong-Yang Liu, Miao Ma

College of Chemistry, Central China Normal University, Wuhan 430079, People's Republic of China

Received 24 February 2009; accepted 30 July 2009
DOI 10.1002/app.31207
Published online 15 September 2009 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: The hydrophobic surface modification of chitosan membranes was performed using the amidating reaction of amino groups on a membrane surface with stearic acid activated by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). The varying surface hydrophobicity of chitosan membranes was achieved by changing the degree of amidation and evaluated by the water contact angle analysis and the adsorption experiment of the hydrophobic dye, Rose Bengal. The effects of the surface hydrophobicity of chitosan membranes on the adsorption behaviors, activity and stability of Candida rugosa lipases were investigated. The experimental results suggested that the increased surface hydrophobicity of chitosan membranes improved the adsorption capacity and activity of the immobilized lipase. The modified chitosan membranes with 30.36% amidation exhibited the maximum activity retention of 83.87%. In addition, a desirable thermal stability was also achieved for the adsorbed lipase. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 115: 1168–1175, 2010

Key words: lipase; chitosan; membrane; surface modification; adsorption

INTRODUCTION

Enzyme-immobilized membranes can function as both catalysis and separation units in enzyme-membrane bioreactors, therefore, have potentials in bioengineering applications. The immobilization of lipase on membrane is important for its practical applications as a versatile biocatalyst for the hydrolysis/synthesis of a wide range of esters.[1] Additionally, chitosan membrane, which has an inherent excellent biocompatibility, has proved to be one of the most promising candidates for enzyme-immobilized materials.[2] However, the hydrophilicity of chitosan seems undesirable for lipase immobilization on account of the property of the interfacial activation of lipases on hydrophobic surfaces.[3] Generally, lipases have some elements of a secondary structure (termed the "lid") covering their active sites and making them inaccessible to substrates. 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 a corresponding functionality for the enzyme. Therefore, the interfacial activation of lipase on hydrophobic surfaces has become a general protocol for lipase immobilization. Although the high activities of lipases via the interfacial activation on hydrophobic surfaces have already been reported by some researchers,[4,5] undesirable enzyme activity loss was sometimes observed.[6] This may be attributed to the fact that the poor biocompatibility of some synthetic hydrophobic supports causes nonbiospecific interactions between the enzymes and materials, protein denaturing and the loss of enzyme activity. Therefore, the hydrophobic modification of biocompatible chitosan materials is necessary for lipase immobilization.

Actually, to our knowledge, the study of the hydrophobic modification of natural materials for lipase immobilization has rarely been reported[7–11] although there were some reported cases of the immobilization of lipase on chitosan materials.[2,12,13] Additionally, our previous studies suggested that the biocompatible interface with hydrophobic groups can help to improve lipase activity.[14–18] These indicate that it is possible to fabricate the hydrophobically modified chitosan membrane materials for lipase immobilization and further develop the high-performance lipase-immobilized membrane bioreactor. Therefore, in this study, the hydrophobic surface modification of chitosan membranes was carried out by the amidating reaction of amino groups on the membrane surface with stearic acid activated

Correspondence to: H.-T. Deng (htdeng2000@hotmail.com).

Contract grant sponsor: Natural Science Foundation of Hubei Province in China; contract grant number: 2006ABA174.

by 1-ethyl-3-(3- dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). The effects of the varying surface hydrophobicity of chitosan membranes caused by the varying degrees of amidation on the adsorption behaviors and activity of Candida rugosa lipases on the nascent chitosan membranes (CS) and stearyl-modified membranes (SCS) were investigated. We hoped that the modified surface of the chitosan membranes, which retains chitosan's inherent excellent biocompatibility, but also facilitates the hydrophobic interfacial activation of lipase, would improve lipase activity to some extent. In addition, this work was also anticipated to lay a foundation for the subsequent development of a high-performance lipase-immobilized membrane bioreactor.

EXPERIMENTAL

Materials

Lipase (from Candida rugosa) was purchased from Sigma. Chitosan (degree of deacetylation $>$ 90%) was purchased from Boou Biotechnology Co. Ltd (Shanghai, PR China). All other reagents were commercially available.

Preparation of chitosan membranes

Chitosan membrane was prepared as previously reported.[19] Chitosan (3 g) was dissolved in 100 mL aqueous acetic acid solution (2 wt %). After stirring for 24 h, the solution was filtered to remove insoluble substances and rested for 5 h to remove the air bubbles. The obtained solution was cast on the surface of a glass plate to form a membrane and dried for 2 h at room temperature. After that, the membrane was immersed in an aqueous NaOH solution (1 mol/L) at room temperature for 24 h and rinsed repeatedly with deionized water. The obtained membrane was crosslinked as reported.[20] The wet membrane was incubating in 0.04 mol/L epichlorohydrin solution (pH 10) at 50°C for 5 h. Thereafter, the resultant membrane was washed extensively with deionized water to remove the residual epichlorohydrin and alkali, and stored in de-ionized water for use. The membranes were cut into circular pieces (diameter: 0.5 cm) with a perforator for the following experiments.

Hydrophobic surface modification of chitosan membranes

In a typical procedure for the surface modification of chitosan membranes,[21,22] 0.05 mol/L 2-morpholino-ethane sulfonic acid (MES, pH 5.5) buffer containing EDC/NHS was prepared with a 2 : 3 molar ratio of EDC to NHS. Meanwhile, stearic acid was dissolved in heated ethanol at a known concentration. Then, two equal volumes of the solutions were mixed well. The molar ratio of carboxyl groups to EDC was 1 : 1.2. The carboxyl-activating reaction was performed at room temperature for 30 min and a solution of EDC/NHS-activated stearic acid was obtained. Before surface modification, chitosan membranes were equilibrated in 0.05 mol/L MES buffer (pH 5.5) for 1 h. A suitable amount of chitosan membranes were incubated in the above solution of EDC/NHS-activated stearic acid. The mixture was shaken gently at room temperature for 24 h. The modified chitosan membranes were removed and rinsed successively with 0.1 mol/L Na_2HPO_4 solution, 4 mol/L NaCl solution and deionized water. The resultant chitosan membranes were stored in deionized water for use. The degree of amidation of the chitosan membranes was controlled by the molar ratio of the carboxyl groups of stearic acid to the amino groups on chitosan membrane surface.

Determination of the surface amidation degree of chitosan membranes

For each experimental datum in the following experiments, in addition to the mentioned in particular, at least three values were averaged to get reliable data, and the standard error for the results was below 5% of the average value.

The surface amidation degree (AD) of the chitosan membranes can be estimated by quantitating the free surface amino group content of the chitosan membranes, and calculated as the following equation:

$$AD = \left(1 - \frac{C_m}{C_n}\right) \times 100\% \quad (1)$$

where C_n and C_m stand for the free surface amino group content of CS and SCS (mol/cm^2 membrane), respectively.

The TNBS (2,4,6-trinitrobenzenesulfonic acid) method[23] was applied to quantitate the free surface amino group content of chitosan membranes. Briefly, an excess of TNBS reagent was added to a dispersion of chitosan membranes in anhydrous ethanol to form TNBS-amine complex after 18 h reaction time. After filtering, the remaining, unbound TNBS in the filtrate was determined by UV absorbance at 340 nm comparing to a standard prepared with DL-valine.

ATR-FT-IR analysis of chitosan and modified-chitosan membranes

Fourier transform infrared spectroscopy (FTIR NEXUS, Thermo Nicolet, America) with an

attenuated total reflection (ATR) unit was used to investigate the chemical changes between the nascent and modified chitosan membranes.

Static water contact angle measurements

The static water contact angles of the membrane surface were measured by the sessile drop method at 25°C with a contact angle goniometer (KRUSS DSA10-MK, Germany) equipped with video capture. At least 10 contact angles were averaged to get reliable data.

Determination of relative surface hydrophobicity of chitosan membranes

The relative surface hydrophobicity of chitosan membranes was quantified by determining the extent of adsorption of the hydrophobic dye, Rose Bengal as reported.[24] Increasing surface area of chitosan membranes were dispersed in a buffered solution of Rose Bengal (0.1 *M* Borax, pH 8). After 2 h, the samples were filtered to remove the chitosan membranes and the filtrate was collected for analysis. The amount of Rose Bengal adsorbed by the chitosan membranes was quantified by determining the difference in the initial amount and the amount remaining in solution by comparison to a standard curve of Rose Bengal obtained by UV absorbance at 548 nm. The slopes of the straight lines obtained from a plot of Rose Bengal partition quotient (the ratio of the amount adsorbed and the initial amount) versus membrane surface area were used to measure the degree of surface hydrophobicity. The greater the slope means the greater the relative hydrophobicity.

Immobilization of lipase by adsorption on chitosan membranes

The immobilization of lipase by adsorption on chitosan membranes was performed as previously reported.[14] In brief, a suitable amount of membranes were immersed in lipase solutions (centrifuged to remove any insoluble substances) with different concentrations and pH. After shaking in a water bath at 37°C, the membranes were taken out and rinsed thoroughly with phosphate buffer until no soluble protein was detectable in washings to remove the reversibly adsorbed protein. The concentration of protein in solution was determined by Bradford's method.[25] The amount of adsorbed protein on the chitosan membranes was calculated from the protein mass balance between the initial lipase solution, resultant supernatant and washings.

Activity assay of free and immobilized lipase

The pH stat method with olive oil titrimetric assay[14] was used to evaluate the lipase activity in this work. Briefly, the olive oil emulsion prepared as previously reported[14] 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. One milliliter of lipase solution (1 mg/mL) or suitable amounts of the lipase-immobilized chitosan 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 in the absence of lipase-immobilized chitosan membranes 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. Membrane activity was the number of lipase unit per cm^2 of membrane. Specific activity was defined as the number of lipase unit per milligram of protein. Activity retention value was the ratio of specific activity of immobilized lipase to that of free lipase.

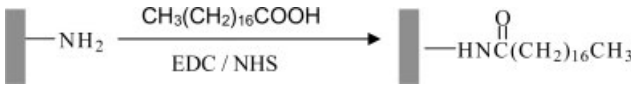
Thermal stability

The free and immobilized lipase preparations were stored in phosphate buffer (0.05 *M*, pH 7.0) at 50°C for 2 h. Samples were periodically withdrawn for activity assay. The residual activity (the ratio of the activity of lipase at a certain incubation time to the initial activity of lipase) was used to evaluate the thermal stability of lipases.

RESULTS AND DISCUSSION

Characterization of chitosan membranes

The hydrophobic surface modification of chitosan membrane is shown as follows.



The degree of amidation of the chitosan membranes was controlled by the molar ratio of the carboxyl groups of stearic acid to the amino groups on membrane surface. The effects of the molar ratio on the amidation degree were shown in Figure 1. It can be seen that the degree of amidation increased with increasing ratio of carboxyl groups to amino groups, and then a plateau value (about 35.54%) was observed after the ratio of 1.5 : 1. Therefore, the chitosan membranes with the changing amidation degrees from 0 to 35.54% (shown in Table I) were chosen for the following experiments.

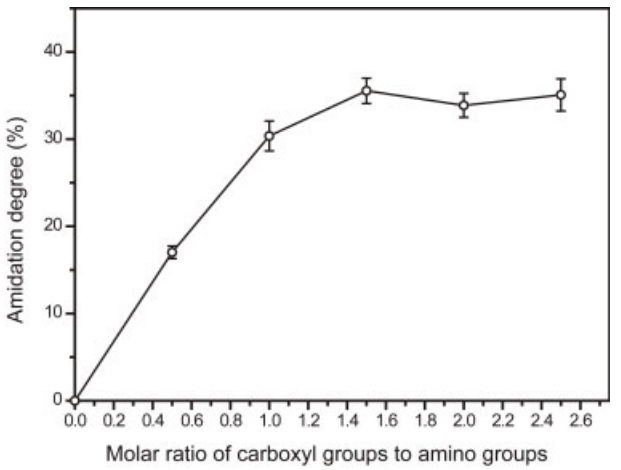


Figure 1 Effect of molar ratio of carboxyl group to amido group on amidation degree.

IR analysis was used to confirm the chemical changes after the modification of chitosan membrane (Fig. 2). CS and SCS-3 was chosen for the characterization. Compared with the IR spectrum of CS, increased peak intensity at about 2846 cm^{-1} and 2919 cm^{-1} was observed for SCS-3. Meanwhile, a strong peak at about 1423 cm^{-1} appeared. These can be ascribed to the introduction of $-CH_2$ and $-CH_3$ groups to chitosan. In addition, after modification, a wide peak was transformed to a double peak in the 1550–1670 cm^{-1} region because the characteristic peak of $-NH_2$ at about 1600 cm^{-1} decreased. Meanwhile, that of amide band I(1665 cm^{-1}) and amide band II(1557 cm^{-1}) clearly increased. These suggested the introduction of the amino groups to the chitosan membrane surface.

In an attempt to investigate the effects of the hydrophilicity/hydrophobicity of membrane surface on the adsorption and activity of lipase, it is necessary to quantify the surface hydrophobicity of chitosan membranes. Here, water contact angle measurement was carried out to evaluate the surface hydrophobiciy of membranes. As shown in Table I, water contact angle increased in order of SCS-3 > SCS-2 > SCS-1 > CS, indicating an increasing surface hydrophobicity with increasing amidation degree.

Further, to verify this changing trend, the relative surface hydrophobiciy of each chitosan membrane was estimated by measuring the adsorption capacities of a hydrophobic dye, Rose Bengal. The linear plots of Rose Bengal partition quotient (the ratio of the amount adsorbed to the initial amount) versus membrane surface area were shown in Figure 3. The slope suggested the degree of surface hydrophobicity. The calculated slopes were shown in Table I. Obviously, the increasing surface hydrophobicity with increasing amidation degree was still observed.

Adsorption behaviors of lipase on chitosan membranes

The time courses of lipase adsorption on CS and SCS were investigated respectively by measuring lipase concentration in solution at different incubation times. The plots of lipase loading as a function of time were shown in Figure 4. The adsorbed amount of protein increased with increasing incubation time, and a plateau value was observed after about 120 min.

The adsorption isotherm plays an important role in predictive modeling for analysis and design of adsorption systems. So, the effects of the initial lipase concentration on its adsorption capacity were investigated and the adsorption isotherms of lipase on CS and SCS were shown in Figure 5.

Among many models established to describe adsorption isotherms, Langmuir model is the simplest and still the most useful isotherm for the sake of convenience.[26] However, the Langmuir model with the ideal assumption such as monolayer coverage of adsorbate over a homogenous adsorbent surface, no interactions existing between adsorbate molecules, etc., is hard to meet the practical process in this case.[27] Therefore, in this work, all the isotherms were fitted to the following Langmuir-Freundlich model[28]:

TABLE I
The Degree of Amidation, Water Contact Angle, Relative Surface Hydrophobicity of CS and SCS, and the Fitting Parameters for the Langmuir-Freundlich Model Fit to the Experimental Adsorption Isotherms of Lipase on CS and SCS

Chitosan membranes	Degree of amidation (%)	Water contact angle (°)	Relative surface hydrophobicity ($mL/\mu m^2$)	q_m ($\mu g/cm^2$)	K	R^2
CS	0	83 ± 3	0.02225	3.54	24.45	0.9968
SCS-1	17.02	86 ± 5	0.03635	3.88	20.96	0.9927
SCS-2	30.36	91 ± 4	0.1318	5.01	16.26	0.9970
SCS-3	35.54	95 ± 5	0.1557	5.40	19.48	0.9856

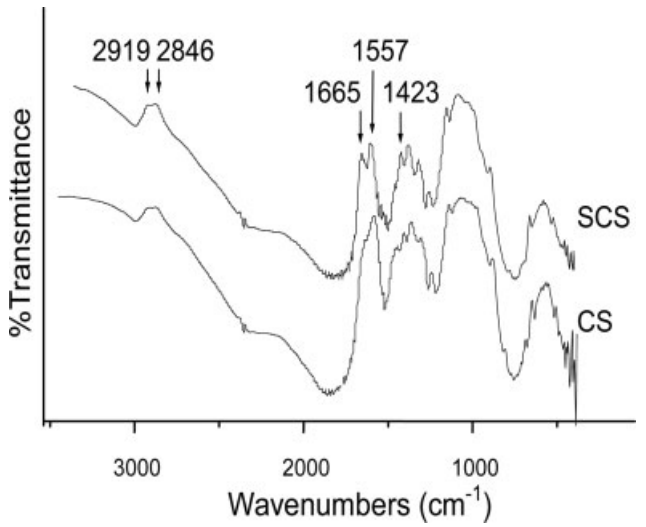


Figure 2 ATR-FTIR analysis of CS and SCS.

$$q = q_m \frac{kc^{1/n}}{1 + kc^{1/n}} \quad (2)$$

where q is the amount of adsorbed lipase at a certain lipase concentration, q_m is the maximum adsorbed amount in equilibrium, C is the bulk concentration of lipase, and k is the adsorption constant. The exponential factor n is relatively constant between 0 and 1, and fixed at 0.5 approximately.

The isotherm model parameters, q_m and k, are evaluated through linearization of Eq. (2):

$$\frac{1}{q} = \frac{1}{kq_m} \times \frac{1}{C^{1/n}} + \frac{1}{q_m} \quad (3)$$

Hence by plotting $1/q$ against $1/C^{1/n}$ it is possible to obtain the value of q_m from the intercept, which is

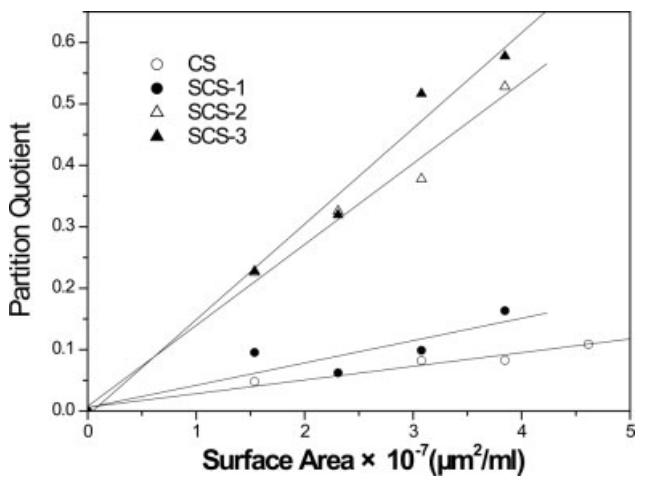


Figure 3 The relative surface hydrophobicity of CS and SCS.

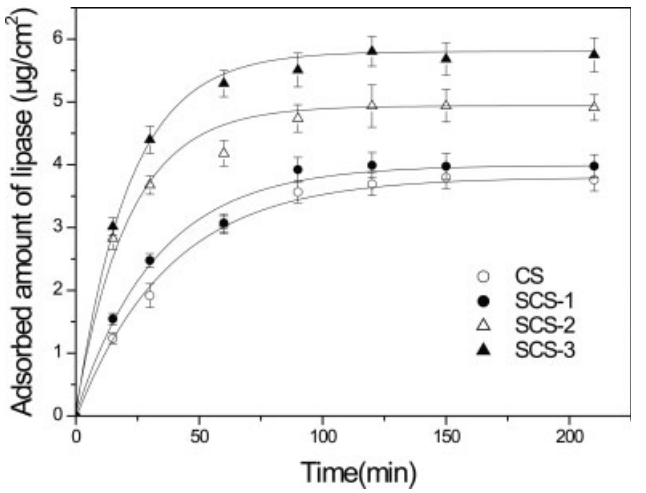


Figure 4 Effect of time on the adsorbed amount of lipase on CS and SCS (pH: 5.0; adsorption temperature: 37°; lipase concentration: 2 mg/mL).

$(1/q_m)$, and the value of k from the slope, which is $(1/k\ q_m)$. Then, the obtained parameters were brought back to the nonlinear equation (2) and the "nonlinear curve fitting" tool in Microcal Origin 6.0 (Microcal Software) was used to fit the scattered datum points in Figure 5 and get the corresponding curve in Figure 5.

These obtained data were listed in Table I. It can be seen that the correlation coefficients suggested the good fitness of Langmuir-Freundlich model for chitosan membranes. Additionally, it was also observed that the maximum adsorbed amount of protein in equilibrium increased with increasing amidation degree and surface hydrophobicity of chitosan membranes. In fact, the same result was also observed in Figure 5.

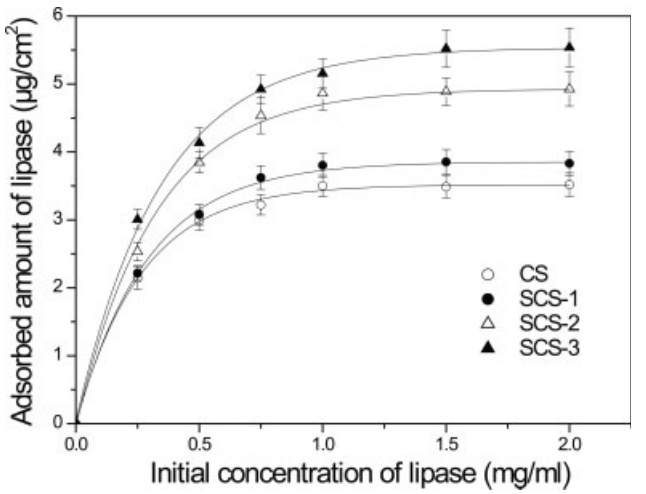


Figure 5 Effect of initial lipase concentration on the adsorbed amount of lipase on CS and SCS (pH: 5.0; adsorption temperature: 37°; adsorption time: 3 h).

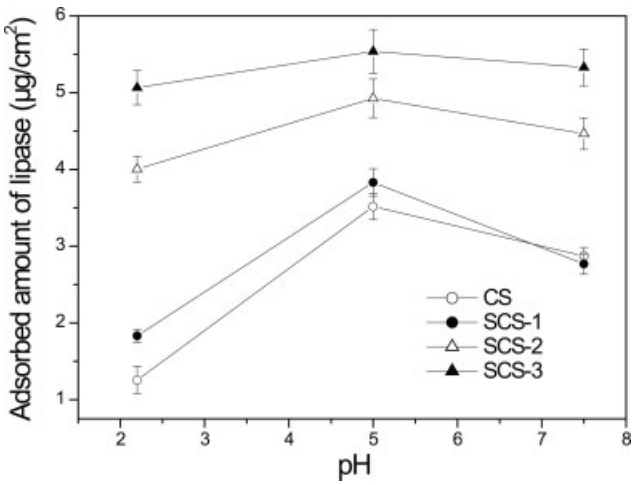


Figure 6 Effect of pH on the adsorbed amount of lipase on CS and SCS (lipase concentration: 2 mg/mL; adsorption temperature: 37°; adsorption time: 3 h).

On the basis of these results, we can assume that the increased surface hydrophobicity by increased amidation degree contributed to the hydrophobic interaction between protein and membrane which accelerated the adsorption process.

In addition, the basically decreasing trend of the adsorption constant K from CS, SCS-1 to SCS-2 was observed in Table I. The similar results were also reported by others and an interpretation was offered.[29] Actually, the physical meaning of K is related to the adsorption kinetics and a higher value of K indicated faster adsorption to reach the equilibrium amount. Obviously, regardless of the influence of the adsorption strength on the adsorption rate, the higher maximum adsorbed amount in equilibrium (q_m) indicated more time needed to reach the adsorption equilibrium, and hereby resulted in the lower K value. However, when the q_m value increased from 5.01 μg/cm^2 of SCS-2 to 5.40 μg/cm^2 of SCS-3, K value increased from 16.26 to 19.48 accordingly inconsistent with the above changing trend. This can be understood as that the enhanced adsorption strength by the stronger hydrophobic interaction between SCS-3 and lipase accelerated the adsorption process significantly, and hereby the effects of the minor gap of only 0.39 μg/cm^2 in q_m value between SCS-2 and SCS-3 on K value can be ignored.

The investigation of the effects of pH on protein adsorption has proven effective in exploring the interaction between protein and support. Therefore, the interaction between lipase and the modified surface of chitosan membranes was estimated by investigating the effects of pH on lipase adsorption on CS and SCS, respectively. The isoelectric point of lipase is around 5.0, so the experiments were performed at pH 2.2, 5.0 and 7.5, respectively. The relevant results were shown in Figure 6. For CS, the maximum adsorption capacity was obtained at pH 5.0 owing to the low lateral repulsion, whereas, at pH 2.2, the adsorbed amount decreased markedly at the electrostatic repulsion between positively-charged lipase and polycationic chitosan membrane. This result indicated that the electrostatic interaction was the main adsorption strength for CS. However, as for SCS-2 and SCS-3, no significant effects of pH on adsorption were observed. This is a typical phenomenon for the hydrophobic interaction between protein and support.[30] Therefore, we can conclude that the hydrophobic interaction between lipase and chitosan has been involved in lipase adsorption after the surface modification of chitosan membranes. This verified the above assumption to some extent.

Effect of hydrophobic surface modification on lipase activity

The activity of lipase on CS and SCS with different amidation degrees was listed in Table II. It was found that the hydrophobic surface modification of chitosan membranes improved the activity of immobilized lipases, and the retention activity increased with increasing amidation in order of SCS-2 > SCS-1 > CS. This result can be ascribed to the changing extent of interfacial activation of lipases caused by the changing degrees of amidation of SCS. As shown in Figure 7, for CS, the adsorption of lipase was dominated by the electrostatic and hydrogen-bonding interaction because of the amino groups and hydroxyl groups on the chitosan membrane surface. Accordingly, when the amount of stearyl groups on the surface increased, the hydrophobic interactions were partially involved in adsorption, and the interfacial activation capacity of lipase increased. For SCS-2 with higher degree of amidation (30.36%), the dense alkyl chains stretched out from chitosan membrane surface and formed a highly hydrophobic layer surrounding the membranes, screening the residual hydrophilic groups and eventually contributing to the hydrophobic interactions between lipase and chitosan. This interaction stabilized the "open state" conformation of the lipase protein. Therefore, SCS-2 exhibited the maximum activity retention of 83.87%. Furthermore,

TABLE II
Activity of Lipase on CS and SCS

Chitosan membranes	Membrane activity (U/cm^2 membrane)	Specific activity (U/mg protein)	Activity retention (%)
CS	0.29 ± 0.014	82.77	67.43
SCS-1	0.34 ± 0.017	88.77	72.32
SCS-2	0.51 ± 0.022	102.95	83.87
SCS-3	0.53 ± 0.023	98.36	80.13

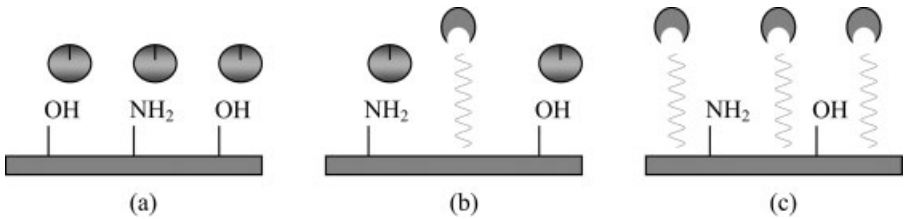


Figure 7 Schematic representative for the interfacial activation of lipase adsorbed on (a) CS, (b) SCS with low substitution degree and (c) SCS with high substitution degree (: membrane; : stearyl group; : open-state lipase; : closed-state lipase).

when the degree of amidation increased from 30.36% (SCS-2) to 35.54% (SCS-3), although the membrane activity still increased, a slight decrease of specific activity or activity retention of lipase was observed. In view of the influence of adsorbed amount of protein on membrane activity, the value of specific activity or activity retention is easer to evaluate accurately the activity of immobilized lipase. This decrease of activity retention may be attributed to the fact that the excessive introduction of stearyl groups to the chitosan membrane surface reduced the original surface biocompatibility to some extent, and led to some non-biospecific interactions between chitosan and lipases. Therefore, we conclude that, although many published reports have verified the hydrophobic interfacial activation of immobilized lipases, a greater hydrophobic interface does not always produce higher activity. In other words, the moderate hydrophilicity/hydrophobicity of the support surface, which favors both the surface biocompatibilty and interfacial activation of lipase, will facilitate the improvement of lipase activity.

Effect of pH and temperature on lipase activity

The effect of pH on the activity of the free and the immobilized lipases was shown in Figure 8. The optimum pH value for the free lipase was about 7.5, whereas those for the immobilized lipases shifted to the alkaline region at around 8.0. It can be explained as that upon immobilization the active site became more exposed to solvent than that in the globular, folded, dissolved lipase form, therefore, proton transfer to the amino acid residues at the active site becomes less hindered.[31]

As shown in Figure 9, the immobilization made the optimum temperature for lipase activity shift from about 35°C of the free enzyme to around 40°C of immobilized lipases. These results can be attributed to a low restriction in the diffusion of the substrates and products at higher reaction temperature and the improved resistance of protein to thermal denaturation.

Thermal stability

The thermal stability of free and immobilized lipases was shown in Figure 10. It can be seen that the free lipase lost all its initial activity within about 90 min. As for the immobilized-lipase the residual activity in 2 h was 40% or so. However, no significant effects of

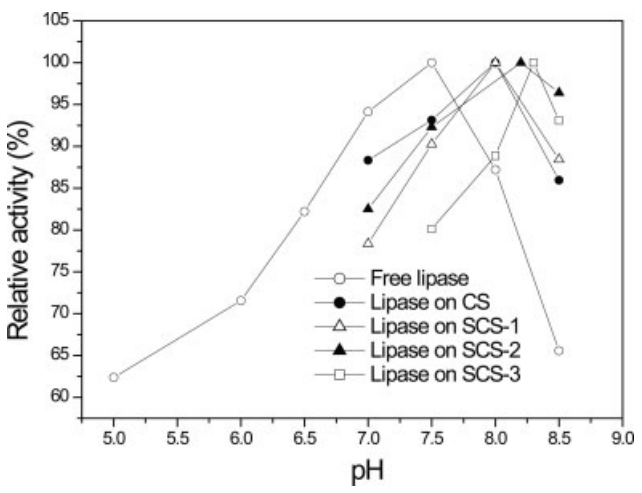


Figure 8 Effect of pH on relative activity of the free and immobilized lipases on CS and SCS.

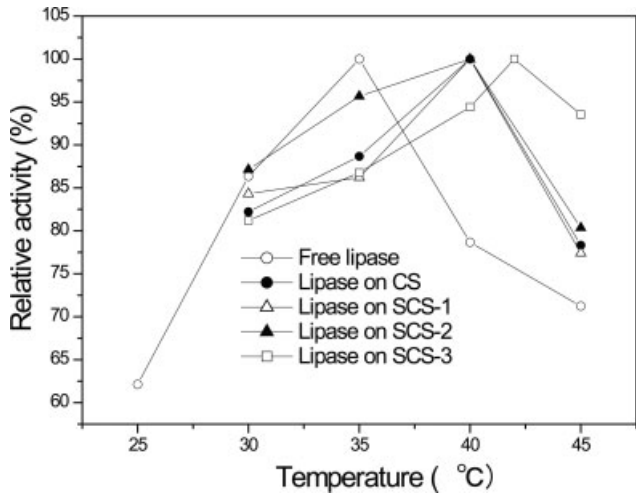


Figure 9 Effect of temperature on relative activity of the free and immobilized lipases on CS and SCS.

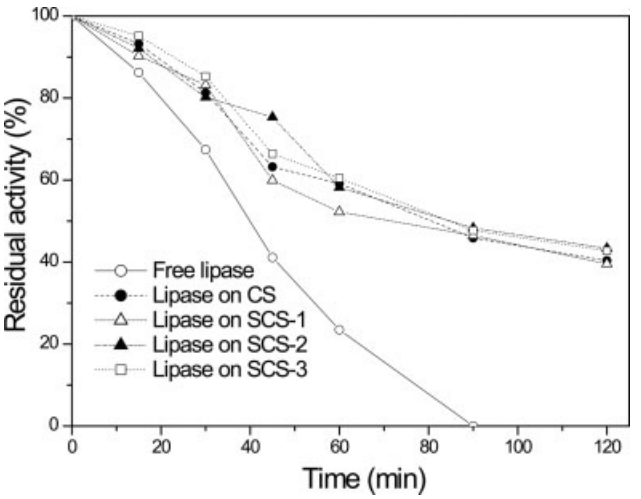


Figure 10 Thermal stability: effect of time on residual activity of the free and immobilized lipases on CS and SCS.

surface modification on thermal stability of lipase were observed.

CONCLUSIONS

The hydrophobic surface modification of chitosan membranes was achieved by the amidating reaction of amino groups on a membrane surface with stearic acid activated by EDC and NHS. The water contact angle analysis and adsorption experiment of hydrophobic dye, Rose Bengal confirmed the increasing membrane surface hydrophobicity with increasing amidation degree. The surface modification of chitosan membranes improved the adsorption capacity and activity of immobilized lipase. SCS-2 with 30.36% amidation that has the moderate hydrophilicity/hydrophobicity benefiting both the surface biocompatibilty of membranes and interfacial activation of lipase, achieved the maximum activity retention of 83.87%.

References

1. Jaeger, K. E.; Reetz, M. T. Tibtech 1998, 16, 396.
2. Krajewska, B. Enzyme Microb Technol 2004, 35, 126.
3. Sarada, L.; Desnuelle, P. Biochim Biophys Acta 1958, 30, 513.
4. Palomo, J. M.; Muñoz, G.; Fernández-Lorente, G.; Mateo, C.; Fernandez-Lafuente, R.; Guisán, J. M. J Mol Catal B 2002, 19, 279.
5. Palomo, J. M.; Segura, R. L.; Fernandez-Lorente, G.; Pernas, M.; Rua, M. L.; Guisan, J. M.; Fernandez-Lafuente, R. Biotechnol Prog 2004, 20, 630.
6. Montero, S.; Blanco, A.; Virto, M. D.; Landeta, L. C.; Agud, I.; Solozabal, R.; Lascaray, J. M.; Renobales, M.; Llama, M. J.; Serra, J. L. Enzyme Microb Technol 1993, 15, 239.
7. Tangpasuthadol, V.; Pongchaisirikul, N.; Hoven, V. P. Carbohyd Res 2003, 338, 937.
8. Rinaudo, M. Prog Polym Sci 2006, 31, 603.
9. Sashiwa, H.; Aiba, S. Prog Polym Sci 2004, 29, 887.
10. Kurita, K. Prog Polym Sci 2001, 26, 1921.
11. Alves, N. M.; Mano, J. F. Int J Biol Macromol 2008, 43, 401.
12. Tang, Z. X.; Qian, J. Q.; Shi, L. Mater Lett 2007, 61, 37.
13. Rodrigues, D. S.; Mendesa, A. A.; Adriano, W. S.; Goncalves, L. R. B.; Giordano, R. L. C. J Mol Catal B 2008, 51, 100.
14. Deng, H. T.; Xu, Z. K.; Huang, X. J.; Wu, J.; Seta, P. Langmuir 2004, 20, 10168.
15. Deng, H. T.; Xu, Z. K.; Wu, J.; Ye, P.; Liu, Z. M.; Seta, P. J Mol Catal B 2004, 28, 95.
16. Deng, H. T.; Xu, Z. K.; Liu, Z. M.; Wu, J.; Ye, P. Enzyme Microb Technol 2004, 35, 437.
17. Deng, H. T.; Lin, Y.; Wang, J. J.; Liu, Z. Y.; Ma, M.; Zheng, F. Surf Rev Lett 2009, 16, 323.
18. Deng, H. T.; Wang, J. J.; Ma, M.; Liu, Z. Y.; Zheng, F. Chin Chem Lett 2009, 20, 995.
19. Santos, D. E. S.; Neto, C. G. T.; Fonseca, J. L. C.; Pereira, M. R. J Membr Sci 2008, 325, 362.
20. Guo, T. Y.; Xia, Y. Q.; Hao, G. J.; Zhang, B. H.; Fu, G. Q.; Yuan, Z.; He, B. L.; Kennedy, J. F. Carbohydr Polym 2005, 62, 214.
21. Park, I. K.; Yang, J.; Jeong, H. J.; Bom, H. S.; Harada, I.; Akaike, T.; Kim, S. I.; Cho, C. S. Biomaterials 2003, 24, 2331.
22. Lee, S. B.; Ha, D. I.; Cho, S. K.; Kim, S. J.; Lee, Y. M. J Appl Polym Sci 2004, 92, 2612.
23. Williams, R. O.; Barron, M. K.; Maria, J. A.; Carmen, R. L. Int J Pharm 1998, 174, 209.
24. Gupta, K. C.; Jabrail, F. H. Carbohydr Polym 2006, 66, 43.
25. Bradford, M. M. Anal Biochem 1976, 72, 248.
26. Ozkaya, B. J Hazard Mater B 2006, 129, 158.
27. Labrou, N. E.; Karagouni, A.; Clonis, Y. D. Biotechnol Bioeng 1995, 48, 278.
28. Yoon, J. Y.; Park, H. Y.; Kim, J. H.; Kim, W. S. J Colloid Interface Sci 1996, 177, 613.
29. Hu, J.; Li, S.; Liu, B. Biochem Eng J 2005, 23, 259.
30. Oh, J. T.; Kim, J. H. Enzyme Microb Tech 2000, 27, 356.
31. Duinhoven, S.; Poort, R.; Van Der Voet, G.; Agterof, W. G. M.; Norde, W.; Lyklema, J. J. Colloid Interface Sci 1995, 170, 340.