Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 55 (2008) 49–54
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Influence of differently modified palygorskites in the immobilization of a lipase

Jianhua Huang, Yuanfa Liu, Xingguo Wang *

The Key Laboratory of Food Science and Safety, Ministry of Education, School of Food Science and Technology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu Province, PR China

Received 9 March 2007; received in revised form 19 December 2007; accepted 19 December 2007
Available online 4 January 2008

Abstract

Lipase, a commercial enzyme, was immobilized onto three different modified palygorskite supports. The palygorskites were modified either by acid treating, or reacting the surface silanol groups present with 3-aminopropyltriethoxysilane, or treating with a quaternary ammonium compound (octodecyl trimethyl ammonium chloride), to produce derivatives with suitable functional group for further utilization in the immobilization of enzyme. The natural palygorskite and their derivatives used were characterized with regard to infrared spectroscopy (FTIR), X-ray diffraction (XRD), surface area and differential scanning calorimetry thermo-gravimetric analysis (DSC–TGA). The amount of lipase adsorbed was much larger on the palygorskite modified by octodecyl trimethyl ammonium chloride (P_{OTMAC}) than that on the acid activated palygorskite (P_a) and the palygorskite modified by 3-aminopropyltriethoxysilane (P_{APTES}). The amount of lipase adsorbed on the P_a was a little smaller than on the P_{APTES}. The enzyme activity and activity recovery in the hydrolysis of olive oil was compared. And, the P_{APTES} showed the highest enzyme activity and activity recovery in the hydrolysis of olive oil. The enzyme activity and the activity recovery of lipase immobilized on P_{APTES} was 27.24 U/g and 19.43%, respectively.

Keywords: Palygorskite derivatization; Lipase; Immobilized enzyme; Enzyme activity

1. Introduction

Lipases are classified as hydrolases that catalyze the breakdown of fats and oils with subsequent release of free fatty acids, diacylglycerols, monoglycerols and glycerol, at a water–oil interface in nature [1]. The enzyme is distributed among higher animals, plants and microorganisms in which it plays a key role in lipid metabolism. Lipase has been applied in many fields including the food industry [2,3], chemistry [4], and the pharmaceutical industry [5,6]. In order to use them more economically and efficiently in aqueous as well as in non-aqueous solvents, their activity, selectivity, and operational stability can be modified by immobilization. The extent of stabilization depends on the enzyme structure, the immobilization method, and the type of support. Numerous supports for the immobilization of lipases have been investigated. Comparative studies indicate that dramatic differences in activity are observed among lipases supported on different materials. Therefore, the support must be chosen with great care. The support can affect the partitioning of substrates, products, and water in the reaction mixture, and thereby, influence the catalytic properties of the enzyme [7]. In previous works, lipases were immobilized by different methods, including covalent attachment to supports [8,9], retaining enzyme on the membrane ultrafiltration surface by filtration [10], cross-linking [11,12], and adsorption [13].

Most globular proteins, such as enzymes, have a strong tendency to accumulate at interfaces. This reactive behavior has a key role in enzyme immobilization and stability. Enzymes are able to be immobilized by clay minerals. Cellulose was absorbed and immobilized on the Avicvel, a soil sample, illite, kaolinite, montmorillonite, and palygorskite. Sorbents was coated with hydroxyaluminum [14]. Montmorillonite clay and clay extracted from Elledge Lake basin

* Corresponding author. Tel.: +86 510 85876799; fax: +86 510 85876799.
E-mail address: wxg1002@hotmail.com (X. Wang).

1381-1177/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2007.12.025

soil were combined with alkaline phosphatase, glucosidase, protease, and xylosidase solutions to assess adsorption and the effect of this adsorption on enzyme activity [15]. The adsorption, desorption, catalytic activity, and susceptibility to microbial degradation of the enzyme horseradish peroxidase, on Wyoming montmorillonite homoionic to Na^+ or Ca^{2+} were investigated [16]. The enzymatic activities resulting from the adsorption of *Rhizomucor miehei* lipase and *Candida cylindracea* lipase onto three different phyllosilicates (sepiolite, palygorskite and montmorillonite) were determined [17]. Candida rugosa lipase non-covalently immobilized on bentonite was given [18]. Natural kaolin as support for the immobilization of lipase from Candida rugosa as biocatalyst for effective esterification was investigated [19]. Activities, stabilities, and reaction kinetics of three free and immobilized α-amylase, β-amylase, and glucoamylase were determined, in which immobilization support was prepared by equal weights of chitosan and activated clay and were cross-linked with glutaraldehyde [20].

Functionalization of carriers is always used to increase the adsorption of protein. The silica sonicated in nitric acid solution, silanized by boiling it in solutions containing different silanes were compared [21]. Immobilization of horseradish peroxidase via an amino silane on oxidized ultrananocrystalline diamond was given [22]. Mercaptopropyltrimethoxysilane and 3-aminopropyltriethoxysilane modified silicons as substrates for protein microarrays were investigated [23]. Esterase immobilization on 3-aminopropyltriethoxysilane bound magnetic nanoparticles through glutaraldehyde linkage was investigated [24]. Nanoporous silicas were functionalized for the immobilization of penicillin acylase [25]. The rationale of this work was to immobilize lipase onto differently modified palygorskite supports, and to obtain the sorption amount of enzyme on the support and the resulting immobilized enzyme activity.

2. Materials and methods

2.1. Materials

The palygorskite used in this work was supplied by Oilbetter Co. (China). Lipase was purchased by the Xueyan Enzyme Co. (China). 3-aminopropyltriethoxysilane (S_{APTES}) was a gift by Shuguang Co. (China). A quaternary ammonium compound, octodecyl trimethyl ammonium chloride (OTMAC) was obtained from the Feixiang Chemical Co. (China). The chemical structures of S_{APTES} and OTMAC were given in Fig. 1. Other chemicals and solvents were obtained from Chinese Chemical Company and were of A.R. grade.

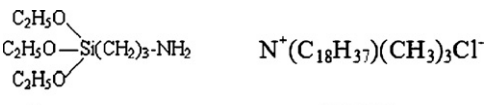

Fig. 1. Chemical structure of S_{APTES} and OTMAC.

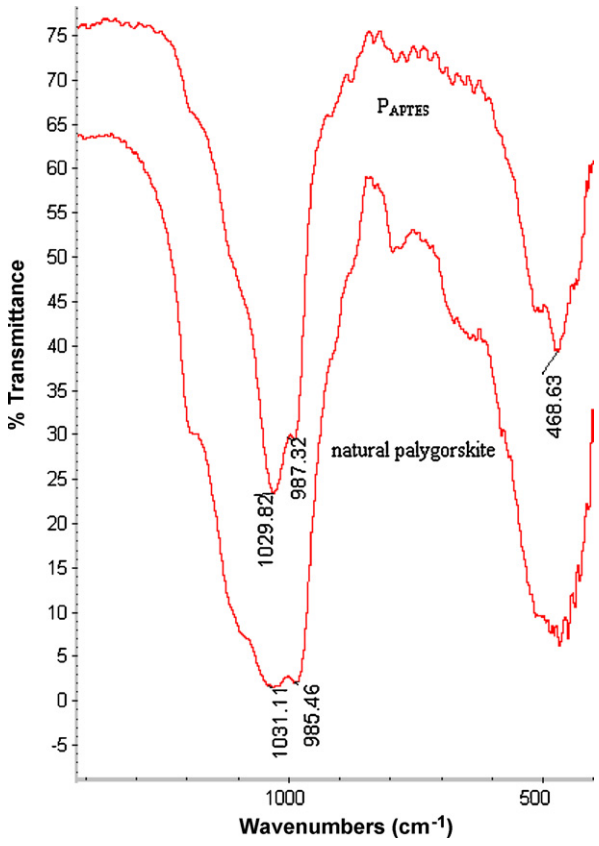

Fig. 2. FTIR spectra of natural palygorskite and P_{APTES}.

2.2. Functionalization and characterization of palygorskite supports

For the creation of hydroxyl groups providing hydrophilic surfaces, the specimens were stirred in dilute HCl solution for 60 min at 50 °C. Excess acid was removed by repeated water rinsing and the acid treated palygorskites were dried. The acid treated palygorskite was denoted by P_a.

The P_a was further modified by 3-aminopropyltriethoxysilane. 10% by volume of 3-aminopropyltriethoxysilane was dissolved in water, after which the pH of the solution was adjusted to 3–4 with concentrated HCl. Samples were immersed overnight at room temperature, removal from the solution over a filter, rinsed with water and dried in vacuo. The percentage of S_{APTES} grafted on the palygorskite was 82%. The P_a grafted with S_{APTES} was denoted by P_{APTES}.

The OTMAC modified palygorskite was prepared according to the methodology reported previously [26]. And the products were denoted by P_{OTMAC}.

The FTIR spectrum of the clay was recorded on a KBr disk, which contains 2% sample by weight, using a Thermo Corporation Nexus FT-IR spectrophotometer (USA). Basal spacing was determined using a Bruker D8S AXS X-ray diffractomer (Germany). The surface area of the clay samples was obtained from the N_2/77K adsorption isotherms by applying the BET method using a Surface Area and Pore Size Analyzer ST-2000B (Beijing Puqi institute of analysis instrument, China). The DSC–TGA analyses were carried out in the temperature range 25–900 °C

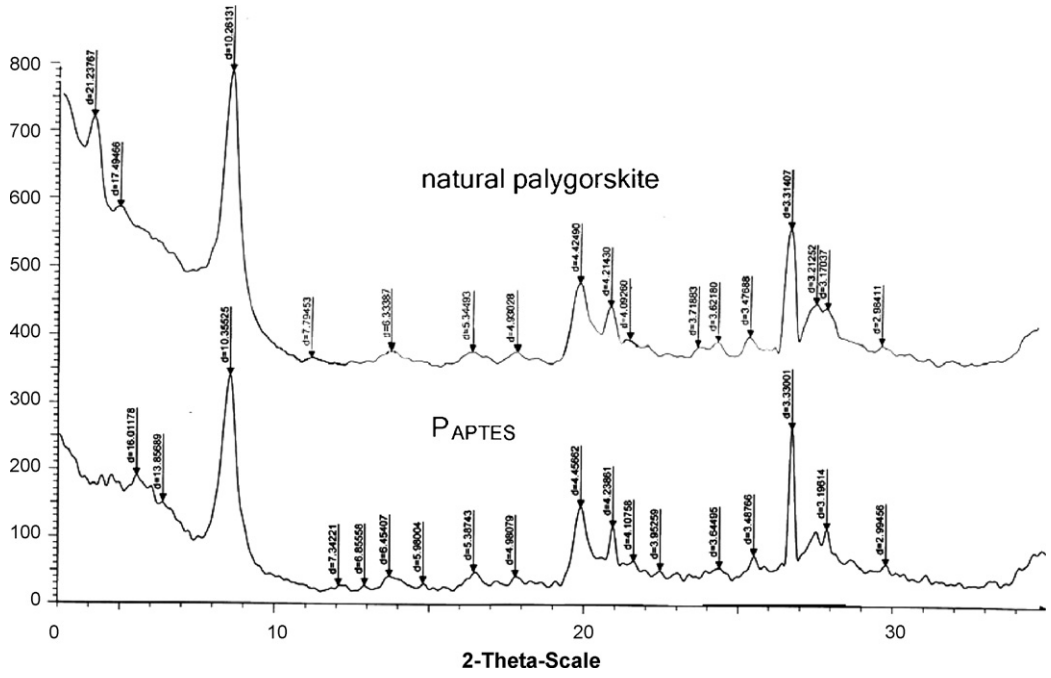

Fig. 3. XRD patterns of natural palygorskite and P_{APTES}.

at a heating rate of 10 °C/min by a thermal analyzer SDT Q600 (TA instruments, USA).

2.3. Methods of immobilization

2.3.1. Immobilization on palygorskite supports

Palygorskite supports were added to 40 ml enzyme solution in 0.1 M phosphate buffer pH 7.5, with a protein content of 1 mg ml^{-1}. The adsorption processes were performed over 12 h at 25 °C with shaking at 110–120 rpm shaking frequency.

2.3.2. Protein content determinations

The protein content in the crude enzyme or immobilized enzyme preparations was determined by the Bradford method, using bovine serum albumin (BSA) as the standard (the protein is, therefore, expressed in BSA equivalents) [27]. The amount of protein bounded onto the palygorskite support was determined indirectly from the difference between the initial total protein exposed to the supports and the amount of protein recovered in the wash.

2.3.3. Enzyme activity assay

The activity of free and immobilized enzyme was assayed by titrating the fatty acid with 0.05 M NaOH. The fatty acid was liberated from the hydrolysis of olive oil under the catalysis of enzymes in phosphate buffer at 37 °C. Activities of the immobilized lipase were assayed by adding 0.1 g of the immobilized lipase in the phosphate buffer, using 5 ml 20% (v/v) olive oil emulsification solution as the substrate, which was obtained after pure olive oil dispersed in water solution containing polyvinyl alcohol (4%, w/v). After exact 15 min of incubation at 37 °C, the reaction was stopped by adding 15.0 ml of alcohol solution (95%, w/w). Finally, the reaction solution was titrated with 0.05 M of hydrochloric acid solution. The blank hydrolysis of olive oil was a same process, except that the alcohol solution was added at the beginning of the hydrolysis. The fatty acid content was calculated from the difference between the blank and acid equation of the titration. One unit of the activity of immobilized lipase was defined as 1 μmol of the fatty acid produced by the catalysis of 1 g immobilized lipase in 1 h under the assay conditions.

3. Results and discussion

3.1. Characterization of modified palygordkites

The natural palygorskite, the P_{OTMAC}, as well as the P_{APTES} were characterized by FTIR and XRD. The FTIR and XRD characterization as well as their surface area of the natural palygorskite and P_{OTMAC} has been previously reported [28]. The FTIR characterization of the natural palygorskite and P_{APTES} was shown in Fig. 2. The bands at 985 and 469 cm^{-1} for the natural palygorskite and P_{APTES} are associated with the bending vibrations of OH and Si–O–Si. The band of 985 cm^{-1} for the natural palygorskite was stronger than that for P_{APTES}. The band of 469 cm^{-1} for the natural palygorskite was weaker than that for P_{APTES}. These observations indicated that the Si–OH of the natural palygorskite was grafted with the 3-aminopropyltriethoxysilane [29]. The XRD patterns of natural palygorskite and P_{APTES} were shown in Fig. 3. The characteristic *d* spacing of 10.31, 4.43, 4.21, and 3.31 Å confirms that the sample used in this work is palygorskite. After graft with 3-aminopropyltriethoxysilane, the *d* spacing for P_{APTES} is 10.36, 4.46, 4.24, and 3.33 Å [28]. The DSC–TGA curves of the natural palygorskite and P_{APTES} were shown in Fig. 4. Four weight loss steps are observed for the natural palygorskite at

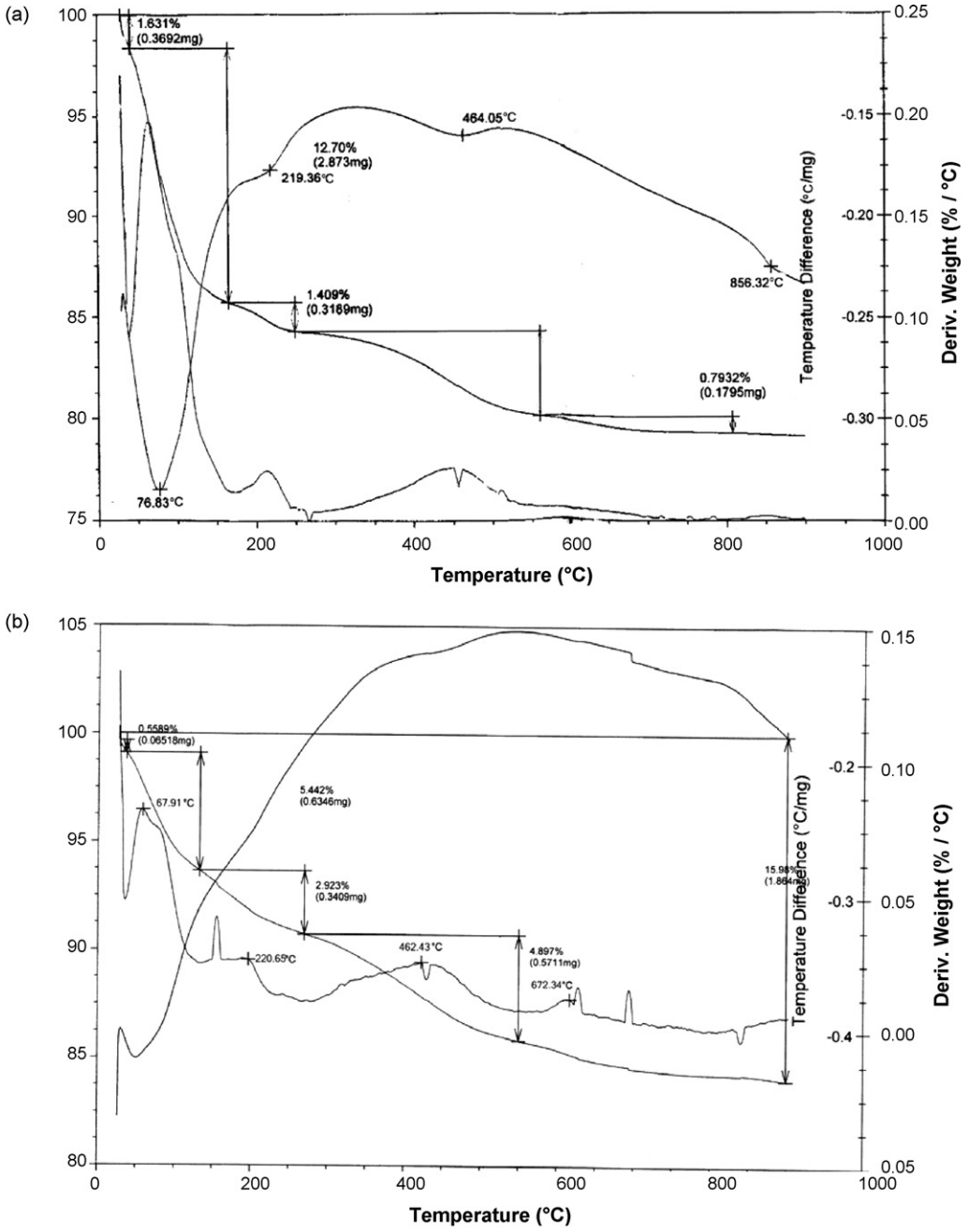

Fig. 4. DSC and TGA curves of (a) natural palygorskite and (b) P_{APTES}.

70, 219, 464 and 856 °C. The weight loss steps are observed for the P_{APTES} at 68, 220, 462 and 672 °C. It is possible to speculate that the last weight loss step is changed for the new structure of the P_{APTES}. The weight loss for each step is also changed after modification by 3-aminopropyltriethoxysilane. According to the results of XRD FTIR and thermal anylsis, it can be concluded that the texture of the palygorskite is maintained and the 3-aminopropyltriethoxysilane is only connected with the surface of palygorskite. The surface area value found to be 182.09 m^2/g for P_{APTES}. The surface areas of nat-

Table 1
Activity and activity recovery of the immobilized lipases

	Loading (mg protein fixed/g support)	Percentage immobilized (%)	Activity (U/g)	Activity recovery (%)
P_a	13.0	59.2	399.6	4.96
P_{APTES}	14.0	63.7	1634.2	19.43
P_{OTMAC}	20.0	90.9	720.5	6.00

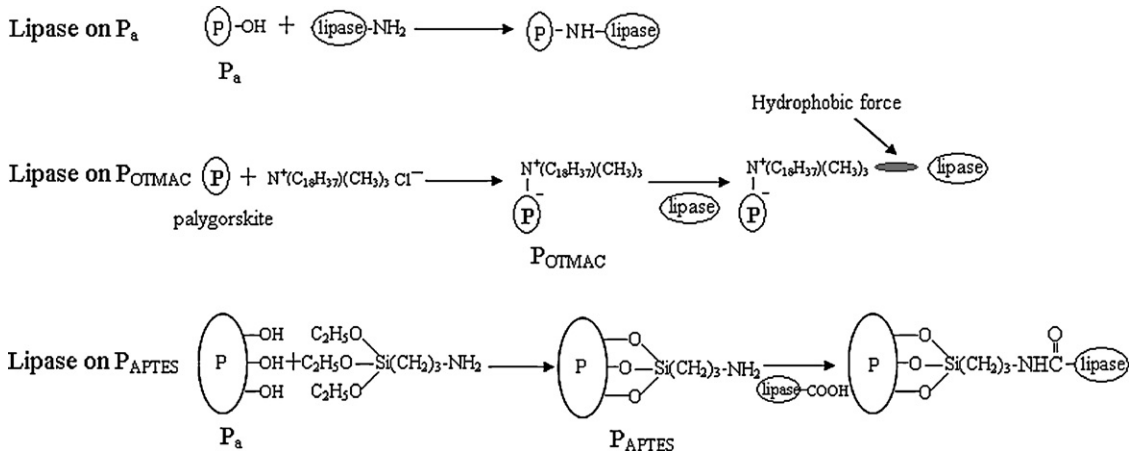

Fig. 5. Schematic illustrations of palygorskite modification and lipase adsorption on the palygorskite derivatives.

ural palygorskite and P_{OTMAC} were 63.96 and 167.24 m^2/g [28].

3.2. Enzyme adsorption

The enzyme uptake on various functionalized palygorskite products was studied. The results showed that the amount of enzyme immobilized on the support P_{OTMAC} was higher than the other two supports (Table 1). The percentage immobilized on the three supports was also given in Table 1. The percentage immobilized on the P_{OTMAC} was 90.9%, which was the highest one of the three. The percentage of lipase immobilized on the palygorskite was lower than 85% reported previously [17]. The support P_{APTES} was found to have little higher enzyme uptake than the P_a. The surfaces with hydrophobic properties were obtained after functionalization of the palygorskites to get the P_{APTES} and P_{OTMAC}. However, the uptake of the protein on the P_{APTES} and P_{OTMAC} was very different. From the literature, no simple rule with regard to the influence of surface wettability on protein adsorption can be deduced. One plausible explanation for the different observations might be based on the employment of different hydrophobizing reagents producing similar degrees of wettability but varying in surface density and orientation of molecule chains which might also be important parameters for protein adsorption [21]. Due to the different nature of the supports used for immobilization, different mechanisms can be expected. Fig. 5 shows the hypothetical illustrations of palygorskite modifications and lipase immobilized to the three supports. We can suppose that for P_a, which are able to promote cationic exchange through silanol residues, the adsorption mechanism must be produced through positive charges (proto-

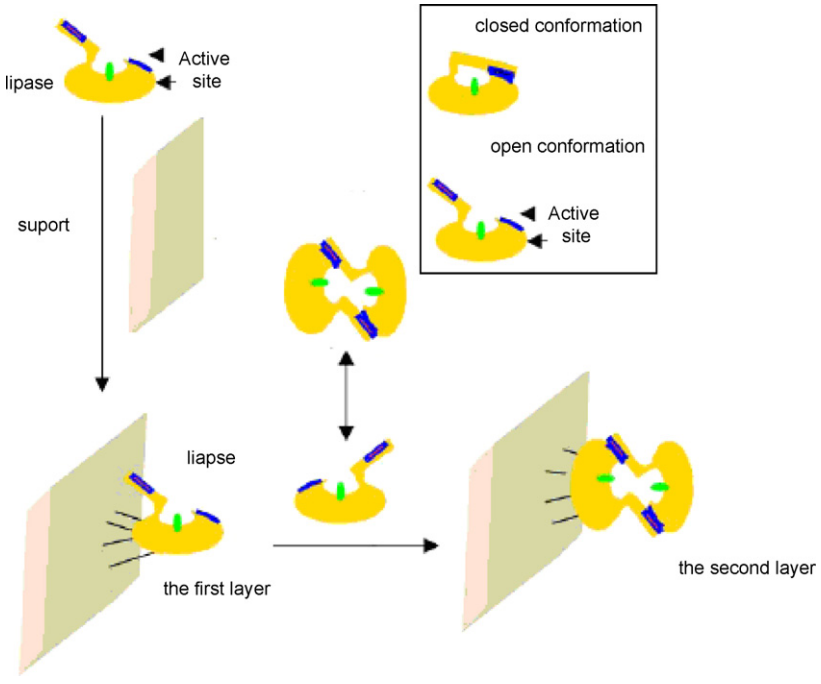

Fig. 6. Schematic illustrations of lipase immobilized on the support P_{OTMAC}.

nated lysines) of the enzymes. For P_{OTMAC}, the hydrophobic interaction between the support and the enzymes was supposed to be responsible for the adsorption. For P_{APTES}, an interaction between the enzymatic carboxylate group and the amino group of the support must be responsible for the adsorption. And the hydrophobic interaction was also existed between the P_{APTES} and the lipase.

3.3. Activities of the immobilized enzyme

For the preparation of immobilized lipase, the activity of the immobilized enzymes on the three supports was shown in Table 1. The activity recovery was also given. The activity recovery of the immobilized enzyme is calculated from the formula:

$$R(\%) = \frac{A}{A_0} \times 100 \quad (1)$$

where R is the activity recovery of the immobilized enzyme (%), A the activity of the immobilized enzyme (U) and A_0 is the activity of the free enzyme in solution before immobilization (U).

The lipase amount adsorbed on the P_{OTMAC} was higher than those on the other two. However, the P_{OTMAC} immobilized lipase present the lower activity and activity recovery to the P_{APTES} immobilized lipase. The lower enzyme activities on P_{OTMAC} supports is likely ascribed to steric impediments (as result of the high content of immobilized enzyme or the longer chain of the quaternary ammonium compound, OTMAC) and to enzyme conformation changes (as result of the multipoint attachment of the enzyme to support). And the other explanation for the lower activity of P_{OTMAC} could be the interference between the first layer lipase adsorbed on the support and the second layer [30]. Fig. 6 shows the illustration of the lipase double-layer adsorption model during the course of lipase immobilization on the support P_{OTMAC}. Moreover, the high content of immobilized enzyme without expressing activity may reflect the immobilization of low-molecular weight contaminant proteins from lipase preparation.

4. Conclusions

Three different modified palygordkites are prepared and characterized. The textures of the three modified palygorskites are maintained and the modifications only receive the different character of the supports surface. For lipase adsorption experiments and olive oil hydrolysis reactions, we have found that 3-aminopropyltriethoxysilane modified palygorskite (P_{APTES}) is a better supports for lipase immobilization than the acid treated palygorskite (P_a) and the OTMAC modified palygorskite (P_{OTMAC}). The different nature of the supports made the different mechanism of lipase immobilization, which made the difference activity and activity recovery of the lipase immobilized on the three products. The higher enzyme adsorption on P_{OTMAC} presented lower enzyme activity.

Acknowledgements

The authors express their gratitude to the NSFC (National Natural Science Foundation of China), for its financial support (Contract NO: 20376028). We also thank the Testing & Analysis Center of Jiangnan University.

References

[1] R. Dalla-Vecchia, D. Sebrão, M.G. Nascimento, V. Soldi, Process Biochem. 40 (2005) 2677–2682.
[2] F.J. Plou, M. Barandiarán, M.V. Calvo, B. Antonio, E. Pastor, Enzyme Microb. Technol. 18 (1996) 66–71.
[3] S. Šabeder, M. Habulin, Ž. Knez, J. Food Eng. 77 (2006) 880–886.
[4] S. Shah, M.N. Gupta, Process Biochem. 42 (2007) 409–414.
[5] J. Quan, N. Wang, X.-Q. Cai, Q. Wu, X.-F. Lin, J. Mol. Catal. B: Enzym. 44 (2006) 1–7.
[6] B.K. Pchelka, A. Loupy, A. Petit, Tetrahedron: Asymmetr. 17 (2006) 2516–2530.
[7] H.F. Castro, P.C. Oliviera, E.B. Pereira, Biotechnol. Lett. 19 (1997) 229–232.
[8] G. Bayramoglu, B. Kaya, M.Y. Arica, Food Chem. 92 (2005) 261–268.
[9] Z.W. Bai, Y. Zhou, React. Funct. Polym. 59 (2004) 93–98.
[10] J. Xu, Y. Wang, Y. Hu, G. Luo, Y. Dai, J. Membr. Sci. 281 (2006) 410–416.
[11] T.-C. Hung, R. Giridhar, S.-H. Chiou, W.-T. Wu, J. Mol. Catal. B: Enzym. 26 (2003) 69–78.
[12] M.L. Foresti, M.L. Ferreira, Enzyme Microb. Technol. 40 (2007) 769–777.
[13] A. Salis, E. Sanjust, V. Solinas, M. Monduzzi, J. Mol. Catal. B: Enzym. 24/25 (2003) 75–82.
[14] A.A.S. Sinegani, G. Emtiazi, H. Shariatmadari, J. Colloid Interface Sci. 290 (2005) 39–44.
[15] T. Tietjen, G.W. Robert, Aquat. Ecol. 37 (2003) 331–339.
[16] I. Lozzi, L. Calamai, P. Fusi, M. Bosetto, G. Stotzky, Soil Biol. Biochem. 33 (2001) 1021–1028.
[17] I.E. de Fuentes, C.A. Viseras, D. Ubiali, M. Terreni, A.R. Alcántara, J. Mol. Catal. B: Enzym. 11 (2001) 657–663.
[18] Y. Yeşiloğlu, Process Biochem. 40 (2005) 2155–2159.
[19] M.B. Abdul Rahman, S.M. Tajudin, M.Z. Hussein, R.N.Z.R. Abdul Rahman, A.B. Salleh, M. Basri, Appl. Clay Sci. 29 (2005) 111–116.
[20] M.-Y. Changa, R.-S. Juang, Enzyme Microb. Technol. 36 (2005) 75–82.
[21] R. Müller, K.-A. Hiller, G. Schmalz, S. Ruhl, Anal. Biochem. 359 (2006) 194–202.
[22] J. Hernando, T. Pourrostami, J.A. Garrido, O.A. Williams, D.M. Gruen, A. Kromka, D. Steinmüller, S.M. Martin, Diamond Relat. Mater. 16 (2007) 138–143.
[23] A.J. Nijdama, M.M.-C. Cheng, D.H. Geho, R. Fedele, P. Herrmann, K. Killian, E. Virginia, E.F. Petricoin III, L.A. Liotta, M. Ferrari, Biomaterials 28 (2007) 550–558.
[24] S.-Y Shaw, Y.-J. Chen, J.-J. Ou, L. Ho, Enzyme Microb. Technol. 39 (2006) 1089–1095.
[25] A.S. Maria Chong, X.S. Zhao, Appl. Surf. Sci. 237 (2004) 398–404.
[26] H. Jianhua, W. Xingguo, J. Qingzhe, L. Yuanfa, W. Ying, J. Environ. Manage. 84 (2007) 229–236.
[27] M.M.A. Bradford, Anal. Biochem. 72 (1976) 248–254.
[28] J. Huang, Y. Liu, Q. Jin, X. Wang, J. Yang, J. Hazard. Mater. 143 (2007) 541–548.
[29] G. Niu, T. Wang, S. Wang, D. Shen, H. Xu, Chin. J. Prosthodont. 3 (2002) 208–211.
[30] J.M. Palomo, C. Ortiz, G. Fernández-Lorente, M. Fuentes, J.M. Guisán, R. Fernández-Lafuente, Enzyme Microb. Technol. 36 (2005) 447–454.